Louisiana State University
LSU Digital Commons

LSU Historical Dissertations and Theses | Graduate School

1992

The Role of Bovine Herpesvirus-1 Glycoprotein III in Molecular Pathogenesis and Immunomodulation.

Katherine Marie Byrne
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
Byrne, Katherine Marie, "The Role of Bovine Herpesvirus-1 Glycoprotein III in Molecular Pathogenesis and Immunomodulation." (1992). *LSU Historical Dissertations and Theses*. 5293.
https://digitalcommons.lsu.edu/gradschool_disstheses/5293

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9301040

The role of bovine herpesvirus-1 glycoprotein III in molecular pathogenesis and immunomodulation

Byrne, Katherine Marie, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1992

Copyright ©1992 by Byrne, Katherine Marie. All rights reserved.

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

THE ROLE OF BOVINE HERPESVIRUS-1 GLYCOPROTEIN III IN MOLECULAR PATHOGENESIS AND IMMUNOMODULATION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences

by
Katherine Marie Byrne
DVM, University of Missouri, 1986
May 1992

DEDICATION

The efforts behind this work are dedicated to the memory of Henry A. Bower, an American farmer. In the middle of the Great Depression, he saw fit to put four daughters through college. He was an inspiration to me in my pursuit of two advanced degrees. It all started with his wonderful stories under a tall farmhouse pine in central Indiana. Thank you, Grandfather.

ACKNOWLEDGMENTS

These investigations were funded by the United States Department of Agriculture Special Research Grants. Louisiana State University Board of Regents provided an Animal Disease Biotechnology Fellowship to support the research efforts in the form of a three year stipend. The School of Veterinary Medicine also provided an SVM Research Fellowship in addition to the Board of Regents Fellowship.

Many people have contributed their time and effort towards completion of this project. Dr. Kousoulas has provided creative scientific thinking. He has provided financial support for this work, and without that support it would not have been possible. Dr. Horohov has provided excellent guidance in the field of immunology. Dr. Corstvet provided the laboratory facilities and excellent support staff. He has also earned my deepest respect and admiration. Dr. Enright furnished the research animals and has been very supportive. Mr. Joel Walker has contributed his time and expertise in animal care and handling. Joel Walker and Fred Enright represent what is best about the veterinary profession--the sincere and generous people. Dr. Todd has always provided thoughtful insight and support. Dr. Amborski served as the only role model for many graduate students within the department.

Dr. Storz has worked to provide excellent facilities and maintain an active research program. Dean Huxsoll and Dean Jenkins have

given valuable guidance and support to all the graduate students at the School of Veterinary Medicine.

The support staff within the Department of Veterinary Microbiology and Parasitology is the best. Jere McBride and Karen McDonough have been unfailingly supportive. Mamie Burrell and Robert Truax have given excellent guidance in cell culture systems. Susan Pourciau and Mary Dixon Powell have been instrumental in the immunologic studies. Rosa Lee Causey has always been great to work with and has done an outstanding job of providing the materials necessary for this work.

The Aquatic Animal Health group has provided friendship and family. Michael Pena has supported my efforts at home and abroad, in all my crisises. Lynn Pittman-Cooley has always listened and made me smile on my darkest day. Larisa Ford has been my friend and companion on the road of higher education. Larry and Lora Hanson are two of my favorite people and have always managed to put my pieces back together. I can not thank them enough for giving me a second family to turn to for help. Jimmy Buffet has provided the appropriate music for dealing with the good times and riches.

Fairie Alice Bower and Mary Byrne, the two strongest, gentlest women I know, have always been by my side and in my heart. Dr. Warren Bower gave me strength when I was at the end. Doris Bower has been inspirational in my pursuit of independence. I will always be indebted to Janet Kimmel for a plane ticket away from the insanity and to the last frontier. I could not have gotten this far without the unconditional support of Maudena Bower.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Bovine herpesvirus-1 (BHV-1) is the pathogenic agent for infectious bovine rhinotracheitis. Current vaccines to protect against infection have serious disadvantages to producers and have been implicated in epizootic outbreaks. The virus has also been shown to cause suppression of the immune system. BHV-1 vaccines could be improved by identifying and genetically deleting those aspects of the virus responsible for immunosuppression and providing a marker within the genome which would distinguish the vaccine strain from wild types in the case of disease outbreaks.

This study examined the role of gIII in viral infection and immunosuppression. This was accomplished by expressing full copy gIII and carboxy-terminal truncations of gIII in a transient eukaryotic expression system. The gene for gIII was then truncated to delete the portion of the protein showing homology to the MHC class II antigen constant domain. Glycoprotein III-derivatives were tested for their antigenic potential in lymphoproliferation assays using bovine peripheral blood mononuclear cells. Truncated versions of gIII lacking the MHC homologous region showed improved antigenic potential. Further studies using the full-copy version of gIII showed it suppressed the proliferative response to mitogen, UV-inactivated BHV-1, and other gIII derivatives.

A gIII-null BHV-1 mutant, KB3305, was isolated to evaluate the effect of deleting the MHC homologous region on the virion and to provide a marker for strain identification. KB3305 replicated 10-fold less efficiently than the wild type and was approximately 15-fold more

cell associated. Heparin reduced the attachment of the mutant to GBK cells by 65%, compared to 75% for the wild type. In addition, KB3305 failed to exhibit hemagglutinating activity, in contrast to the wild-type strain. In lymphoproliferation assays, KB3305 was able to stimulate an equivalent response to that induced by the wild type virus, indicating that the lack of gIII does not influence viral antigenicity.

INTRODUCTION

Respiratory and reproductive diseases combined are the most common cause of disease in cattle (Salman et al., 1991a). Bovine herpesvirus-1 contributes to both disease forms by acting as the causative agent of infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, and infectious abortions. The cost of disease prevention in these cases is much less than the cost of treatment and loss of diseased animals from death and culling (Salman et al., 1991b). Immunization is the most direct method of disease prevention.

Several vaccines for bovine herpesvirus are currently available and are typically composed of attenuated live virus given intranasally or intramuscularly. However, these vaccines have serious side effects and complications. Intramuscular vaccine versions can produce abortions in pregnant cattle (Van Der Maaten, Miller, & Whetstone, 1985b). Vaccine strains have also been implicated in disease outbreaks (Whetstone, Wheeler, & Reed, 1986). This has resulted from the fact that attenuated live vaccine strains can be transmitted to non vaccinates and are capable of establishing latent infections (Baker, Rust, & Walker, 1989; Pastoret et al., 1980). Current inactivated vaccines do not provide adequate protection (Frerichs et al., 1982).

Attenuated live strains are typically isolated by culturing the virus in extraordinary conditions. As implied by the occurence of epizootic outbreaks, attenuated vaccine strains can revert to virulence in the host. Recent developments in the area of recombinant genetics and molecular biology provide an alternative production method for new vaccine strains. Recombinant strains can be designed which

have virulence factors deleted, or genetic markers inserted into the genome. Herpesviruses are good candidates for such manipulations because of their large genomes. Viruses can also be used as vectors to impose the expression of heterologous antigens by the infected host cells (Desrosiers et al., 1985; Clark, 1987). The large genome of herpesvirus is an advantage in this case as well by allowing larger genes to be inserted.

Bovine herpesvirus-1 is a well qualified candidate for genetic manipulation. Deleting a gene encoding a protein expressed on the surface of infected cells would provide a readily identifiable marker to distinguish outbreaks caused by the vaccine strain. In addition, bovine herpesvirus has demonstrated immunosuppressive qualities (Babiuk, Lawman, & Greiebel, 1989). If specific proteins or epitopes contributed to the suppressive effect, they could be deleted from the gene while maintaining other antigenic areas on the protein. This project has focused on the identification of viral proteins which might be responsible for bovine herpesvirus-1 immunosuppression and the isolation of a mutant virus which does not express them.

CHAPTER 1

Literature Review

GENETIC CHARACTERISTICS OF BOVINE HERPESVIRUS-1

Bovine herpesvirus-1 (BHV-1) belongs to the family Herpesviridae. It is classified in the Alphaherpesvirinae subfamily, indicating it produces a rapid infection which results in cell lysis and acute infectious disease. Alphaherpesviruses characteristically establish latent infections in ganglion cells after initially causing epithelial lesions. Five different subtypes of BHV-1 (1, 2a, 2b, 3a, 3b) have been identified based on their restriction endonuclease patterns, viral protein characteristics, and reactivity with monoclonal antibodies. (Wyler, Engels, & Schwyzer, 1989). The molecular differences in subtypes are expressed in their most common clinical manifestations. Subtypes are not, however, rigidly defined by their clinical signs. Subtype 1 isolates have typically been recovered from infectious bovine rhinotracheitis cases . Subtypes 2a and 2b have been recovered from infectious pustular vulvovaginitis cases. Subtypes 3a and 3b show affinity for neural tissues, having been isolated from encephalitis cases in Australia and Argentina.

The bovine herpesvirus-1 virion consists of a double strand of DNA surrounded by an icosahedral nucleocapsid which is in turn enveloped within a host-derived lipid bilayer (Ludwig & Letchworth, 1982). The proteinaceous tegument lies between the capsid and the envelope. The viral genome of the Cooper strain is approximately 136.9 kilobases long (Mayfield et al., 1983) but published sequences for all BHV-1 strains have varied from 133.3 kilobases (Engels, Steck, &

Wyler, 1981) to 139.5 kilobases (Engels et al., 1986). Viral DNA has a high G-C content equivalent to 72% of the bases (Ludwig, 1982). The genome is composed of two parts: the unique long and the unique short regions. Because of the inverted repeats bracketing the unique short region, the genome can exist as two isomers making it a member of the group D herpesvirus DNA. The origin of replication for the genome has been characterized and used independently for plasmid production (Westerman & Skare, 1990; Carpenter & Misra, 1991)

Gene expression from the BHV-1 genome is sequentially ordered and can be grouped into immediate early (alpha) proteins, early (beta) proteins, and late (gamma) proteins. The expression of each set is dependent on the successful progression of events in the infection and reproduction of the virus. Immediate early genes do not require the expression of other viral genes. Early protein synthesis is dependent on the expression of immediate early genes. Late protein synthesis requires expression of immediate early and early proteins as well as viral DNA synthesis (Roizman et al., 1974; Misra, Blumenthal, & Babiuk, 1981; Ludwig & Letchworth, 1987, Wirth et al., 1989) Four glycoproteins have been described for BHV-1. They are gI, gII, gIII, and gIV. Of these four, gI, gIII, and gIV are considered to be the most significant based on their prominent contributions to the pathogenesis of infection and the host's immune response to the virus. Three other glycoproteins-gh, gx, and gi-may also exist, but have not been well characterized (Schwyzer, 1991; Baranowski et al., 1991). The virus also encodes for a thymidine kinase (Weinmaster et al., 1982), a DNA polymerase (Owen & Field, 1988), a major DNA

binding protein (Bandyopadhyay, Mittal, & Field, 1990), a major tegument protein (Carpenter & Misra, 1990), a latency related protein (Hayes & Rock, 1989), and at least three immediate early transcripts-IE1,IE2,and IE3 (Wirth et al., 1989).

MAJOR GLYCOPROTEINS OF BOVINE HERPESVIRUS-1

Glycoprotein I of BHV-1 is the gB homolog of HSV-1 and is expressed as an early protein (Misra, Nelson, & Smith, 1988; Whitbeck, Bello, & Lawrence, 1988). It is essential for growth in cell culture (Wyler , Engels, & Schwyzer, 1989). It is located in the Hind III "A" fragment of the viral genome and its sequence is known (Lawrence et al., 1986; Whitbeck, Bello, & Lawrence, 1988). It exists as a cleaved, disulfide-linked heterodimer embedded in the viral envelope (Van Drunen Littel-Van Den Hurk & Babiuk, 1986). The 130K mature product is cleaved to a 74K and 55K linked heterodimer. Cleavage of the protein is not essential for its function (Blewett & Misra, 1990). It contains N-linked glycosylation which has been found to be important for serum neutralizing antibody response (Van Drunen Littel-Van Den Hurk, Hughes, & Babiuk, 1990b; Van Drunen Littel-Van Den Hurk & Babiuk, 1985c; Whitbeck, Bello, & Lawrence, 1988). It is a major target for the humoral and cellular immune response of infected cattle (Hutchings, Van Drunen Littel-Van Den Hurk, Gifford, & Babiuk, 1990a; Marshall, Israel, & Letchworth, 1988). Glycoprotein I has been shown to have a secondary role in viral attachment (Liang et al., 1991a). When independently expressed in a cell culture system, gI induced spontaneous fusion (Fitzpatrick, Zamb,

& Babiuk, 1990b); gI may also interfere with the cytopathic effect of BHV-1 infection (Chase, Carter-Allen, & Letchworth, 1989).

Glycoprotein III is the gC homolog of HSV-1, and is expressed as a late protein (Misra, Blumenthal, & Babiuk, 1981). The gene encoding gIII has been mapped and sequenced. From this sequencing effort, gIII was found to have three domains which mimicked the immunoglobulin superfamily (Fitzpatrick, Babiuk, & Zamb, 1989). One domain in particular showed 68% homology over 96 amino acids to the MHC class II antigen constant domain. Antigenic mimicry by gIII to a cell-surface glycoprotein on bovine macrophages, polymorphonuclear leucocytes, and platelets was also demonstrated by identifying cross-reacting monoclonal antibodies. In addition, gIII is also able to bind bovine complement component C3 and thereby protect itself from complement-mediated injury (Huemer et al., 1991).

Glycoprotein III has a mature molecular weight of 91K and exists as a homodimer on the virion envelope (Van Drunen Littel-Van Den Hurk, & Babiuk, 1986). It has been found to be non-essential for growth in cell culture (Liang, Babiuk, & Zamb, 1991b; Nelson et al., 1989) and to contain both N- and O- linked glycosylation sites (Van Drunen Littel-Van Den Hurk, & Babiuk, 1985c). Both the humoral and cellular immune responses recognize gIII as a major target, although gIII was less immunogenic of the three major glycoproteins which were compared (Hutchings, Van Drunen Littel-Van Den Hurk, Hughes, & Babiuk, 1990b; Marshall, Israel, & Letchworth, 1988; Van Drunen Littel-Van Den Hurk, Gifford, & Babiuk, 1990a). Glycoprotein III has been shown to be responsible for the hemagglutinating activity of BHV-1 (Trepanier et al., 1985; Trudel et

al., 1987). It has been shown to have a predominant role in viral attachment (Liang et al., 1991a) and that this attachment is sensitive to the presence of heparin (Okazaki et al., 1991). Only gIII was shown to bind heparin in these studies. In other herpesvirus systems, both the gC and the gB homologs were shown to interact with heparin (Mettenleiter et al., 1991; Herold et al., 1991). The implication of this observation is that herpesviruses may initially bind to heparan sulfate, a ubiquitous cell surface proteoglycan, using a gC homolog. This mechanism may serve to concentrate the virus on the cells' surface for easier access to a secondary receptor for penetration (WuDunn & Spear, 1989).

Glycoprotein IV is expressed as an early protein and is the gD homolog of HSV-1 (Tikoo et al., 1990). It has been found to be essential for viral replication and responsible for penetration and egress (Fehler et al., 1991). The mature glycoprotein weighs 71K and exists on the virion envelope as a homodimer (Van Drunen Littel-Van Den Hurk & Babiuk, 1986). However, researchers have been able to identify size differences between strains. These variations can yield a protein as large as 84K (Keil, Weidemann, & Beninga, 1989). Glycoprotein IV contains both N- and O- linked glycosylation sites (Van Drunen Littel-Van Den Hurk & Babiuk, 1985c). Neutralizing antibodies directed towards gIV are not dependent on the glycosylation of the protein (Van Drunen Littel-Van Den Hurk, Hughes, & Babiuk, 1990b). Glycoprotein IV is a major target for the humoral and cellular immune response of the host (Hutchings, Van Drunen Littel-Van Den Hurk, Hughes, & Babiuk, 1990b; Marshall, Israel, & Letchworth, 1988; Van Drunen Littel-Van Den Hurk,

Gifford, & Babiuk, 1990a). In these studies, gIV proved to be the most potent immunogen of the three major glycoproteins. The gene for gIV has been mapped and sequenced, and the protein expressed independently (Tikoo et al., 1990; Van Drunen Littel-Van Den Hurk et al., 1991; Chase et al., 1990). Using eukaryotic cells to express gIV on the surface, it was shown that these cells could resist infection by BHV-1 (Chase et al., 1990).

VIRAL PATHOGENESIS AND LATENCY

Transmission of BHV-1 occurs via the secretions of the respiratory, ocular, and reproductive tracts of infected animals. In addition to natural infection, cattle can also be infected by contaminated semen at the time of artificial insemination (Bielanski, Loewen, & Hare, 1988). After entering the host, BHV-1 multiplies at the site of entry in the mucosal and submucosal cells (Yates, 1982). At this time, the virus also gains entry into peripheral nerves. A transient viremia occurs, but systemic dissemination is believed to occur via infected leukocytes (Cummins & Rosenquist, 1977; Castrucci et al., 1980; Rossi & Kiesel, 1977; Nyaga & McKercher ,1980). From the initial site of entry, the virus can be transmitted to the brain to cause encephalitis, to the upper airways to cause bronchitis, or to the ovaries and developing fetus to result in abortion.

Latency is established via peripheral nerve endings and persistance of the virus in the trigeminal ganglion (Narita et al., 1976; Homan & Easterday, 1980). Reactivation occurs with stress and can be induced with exogenous immunosuppressants such as dexamethasone. Two different strains of BHV-1 can also establish

simultaneous latency within a host (Whetstone & Miller, 1989a). Genetically altered BHV-1 strains are still able to establish latency despite being temperature sensitive or lacking the thymidine kinase gene (Kit et al., 1985; Pastoret et al., 1980). On a molecular level, only a 1.16 kilobase region of the Hind III "D" fragment of the genome hybridized to latently infected neurons, indicating transcription is active but restricted in latent infections to the latency-related transcript (Rock, Beam, & Mayfield, 1987). Furthermore, antibodies developed from peptides produced from the open reading frames in this region identified a protein doublet of 33-35K and that the protein was present in purified virions (Hayes & Rock, 1989). The promoter for the latency-related transcript has been found to be very active in ganglionic neurons but less so in other cells. In addition, removal of the "silencer" region of the promoter enabled the promoter to function in bovine cells (Jones et al., 1990).

Immunosuppression by bovine herpesvirus contributes to the pathogenesis of the infection and aids the establishment and maintenance of latency. BHV-1 influences the immune system in many ways (Babiuk, Lawman, & Griebel, 1989). Infection in vivo or in vitro has been shown to reduce the mitogenic response of cultured bovine lymphocytes (Babiuk & Ohmann, 1985; Filion, McGuire, & Babiuk, 1983; Carter et al., 1989; Ghram et al., 1989). Live and UV-inactivated BHV-1 has also been shown to reduce the reactivity to antigen and interleukin-2 (Hutchings et al., 1990a). BHV-1 also interferes with immune cell functions. Infected macrophages have shown reduced Fc-mediated receptor acitvity, reduced phagocytosis and reduced antibody-dependent cell cytotoxicity (Forman & Babiuk,

1982). Following infection, the chemotactic response of polymorphonuclear cells is reduced (Babiuk & Ohmann, 1985; Filion, McGuire, & Babiuk, 1983). BHV-1 also affects the production of lymphokines. Interleukin-2 levels are reduced after infection as well as the production of chemotactic factor by macrophages (Babiuk & Ohmann, 1985). Addition of exogenous interleukin-2 could not restore proliferation to mitogen or antigen (Hutchings et al., 1990a; Babiuk & Ohmann, 1985). Other researchers have investigated the BHV-1 response in low responder cattle. Their results, using a limiting dilution frequency analysis, suggest the presence of suppressor cells (Miller-Edge & Splitter, 1986).

MOLECULAR MIMICRY AND HERPESVIRUSES

The term molecular mimicry identifies similar protein or DNA sequences, or similar antigenic qualities between two dissimilar genes or their protein byproducts (Damian, 1989; Fujinami & Oldstone, 1985; Fujinami et al., 1983). As indicated in the definition, molecular mimicry is identified by comparing the protein and DNA sequences, or by using panels of monoclonals to find cross-reacting epitopes. Mimicry to host proteins by pathogenic organisms has been proposed to provide an immunologic advantage for the invading microbe by enabling them to evade the host's surveillance by disguising themselves as 'self'. In addition, the proteins demonstrating mimicry could also behave as a functional host protein or indirectly influence the action of the host protein by competitively inhibiting its action. Autoimmunity is also an indirect consequence of molecular

mimicry. It does not provide an advantage for the virus, but has serious consequences for the host suffering a chronic viral infection.

Several herpesviruses have shown molecular mimicry in their proteins. Epstein-Barr virus protein gp350/220 has a similar amino acid sequence with the complement C3dg fragment. Both proteins attach to the CR2 receptor on B and T lymphoblasts (Nemerow et al., 1989). In this case, EBV has mimiced the complement fragment to gain entry into a select cell line. The BCRFI gene of EBV also has protein sequence homology to the host's IL-10, the cytokine synthesis inhibitory factor (Moore et al., 1990). Both IL-10 and the BCRFI protein inhibit interferon-gamma synthesis. The mechanism of this mimicry appears to be that EBV uses this homology to mimic the cytokine function and influence the immune response. Human cytomegalovirus also shows significant mimicry to the MHC class I molecule (Beck & Barrell, 1988). The UL18 open reading frame protein product has been shown to functionally mimic the MHC I molecule by binding Beta2-microglobulin. It is suspected that this mechanism either disguises the UL18 viral protein, or prevents the maturation of cellular MHC I molecules. Without mature MHC I molecules on their surface, infected cells are unrecognizable to cytotoxic T cells (Browne et al., 1990).

CLINICAL DISEASE

As seen with the various subtypes, BHV-1 infection can cause a variety of clinical syndromes. It more commonly results in infectious bovine rhinotracheitis (IBR), but can also cause abortions, infectious pustular vulvovaginitis (IPV), balanoposthitis, conjunctivitis, and

encephalitis. It is also a significant component of the bovine respiratory disease complex which incorporates a number of pathogens.

Infectious bovine rhinotracheitis is an upper respiratory tract infection characterized as a laryngotracheobronchitis. Clinical signs include pyrexia, increased respiratory rate, dypsnea, persistant harsh cough, anorexia and emaciation. Diarrhea can occur in systemically infected animals, but is unusual. Dairy cows show a dramatic decrease in milk production. Conjunctivitis and excessive ocular discharge can also accompany the respiratory form of the disease. Profuse nasal discharge occurs early in infection and proceeds from clear to mucopurulent. The nasal mucosa is usually reddened and hyperemic, with white nasal plaques visible on the surface. Histologically, the epithelium of the upper airways is necrotic, with destruction of the mucociliar system. Dyspnea resulting from blocked airways, may cause infected animals to open-mouth breathe. Auscultation of the lung field in cases of IBR may reveal a tracheitis, but the lung field itself remains clear. If advanced lung sounds are heard, it is indicative of other complicating pathogens or factors. (Kahrs, 1977; Wyler, Engels, & Schwyzer, 1989; Blood & Radostits, 1989).

The role of BHV-1 in pneumonia is often a point of confusion in the differentiation of disease caused by uncomplicated BHV-1 versus the multifactorial agents comprising the bovine respiratory disease complex. Clinically, pneumonia which occurs with IBR results from secondary pathogens, primarily bacterial invaders. In a recent review of IBR, Yates indicated that experimental studies show BHV-1 may

contribute to lung lesions independent of other pathogens (Yates, 1982). However, the experiments cited are still complicated by the isolation of secondary pathogens (Markson & Derbyshire, 1966), unsatisfactory experimental animals (McIntyre, 1954), lack of bacterial culture follow-up on pneumonic lungs (Shroyer & Easterday, 1968), and inappropriate inoculum (McKercher et al., 1955). The mechanical effects of dyspnea associated with IBR congestion also complicate the singular effects of the virus by transporting infective debris to the lower lung fields and there by contributing to lung damage. The precise role of BHV-1 in lung pathology has been difficult to distinguish and not clearly understood. The bulk of evidence and consensus of reviewers indicates uncomplicated BHV-1 infection does not produce pneumonia.

Abortions may accompany the respiratory form of BHV-1 infections but can also follow any of the clinical forms of BHV-1 as well as asymptomatic infections within an infected herd. A quarter of pregnant cattle may abort following an outbreak (Kahrs, 1977). Cattle between the 5th and 9th month of pregnancy are most often affected. A typical infectious scenario will begin with an acute outbreak of respiratory disease lasting 5 to 10 days. Some abortions may occur while clinical signs are present in the herd, or may be delayed for up to 100 days following the initial outbreak. There are several outcomes for the affected fetus. It may be mummified, stillborn, die in utero several days before expulsion, or result in a weak calf showing signs of IBR and lesions along the alimentary tract. Microsopic lesions can sometimes be found in the autolyzed fetus

and include focal necrosis of the liver, kidneys, adrenal glands, and stomach.

BHV-1 infection of the genital tract results in infectious pustular vulvovaginitis and balanoposthitis. Cases usually occur after natural mating with an infected animal. Painful conditions cause frequent micturition and an elevation of the tail. The surface of the vulva shows white plaques consisting of necrotic material and the presence of a mucopurulent discharge. Rarely are outbreaks of combined respiratory and genital forms of BHV-1 seen occuring together. (Gibbs & Rweyemamu, 1977). This could be explained by different tissue tropisms of the two different subtypes. The occurrence of two clinical forms together would represent a superinfection by the second subtype.

The third subtype of BHV-1 has been shown to cause meningoencephalitis in calves (Brake & Studdert, 1985; Metzler, Schudel, & Engels, 1986). The presenting clinical signs include incoordination and circling, muscular tremors, ataxia, and blindness. Outbreaks of BHV-1 encephalitis occur sporadically.

Conjunctivitis is the final more common clinical manifestation of BHV-1 infection. Pustules and plaques of necrotic debris may be seen on the conjunctiva, but do not always develop. Corneal opacities can develop from the scleral junction. This is a differentiating point from Moraxella bovis pinkeye which develops corneal opacities from the corneal center outwards.

The prevention of viral diseases is dependent on effective vaccines. Current vaccines for BHV-1 are hampered by ineffectiveness, and adverse side affects. Present vaccines on the

market consist of either killed or attenuated live virus. They are typically administered intra-nasally or intra-muscularly. Attenuated live vaccines are more commonly used because of the questionable effectiveness of vaccine versions comprised of killed virus (Frerichs et al., 1982). Live vaccine versions have their own set of complicating side effects. Live vaccine strains of BHV-1 are still able to establish latent infections as is characteristic of herpesviruses (Pastoret et al., 1980). Transmission of attenuated live vaccine virus to nonvaccinates can also occur and is further complicated by reactivation of latently infected vaccine strains (Baker, Rust, & Walker, 1989; Whetstone, Miller, & Van Der Maaten, 1989b). Vaccine strains have also been implicated in epizootic outbreaks (Whetstone, Wheeler, & Reed, 1986). Intra-nasal vaccines were developed to overcome the abortive effects of the intra-muscular version. Systemic inoculation of attenuated live virus can lead to ovarian lesions which in turn decrease the progesterone concentration and can result in abortion (Van Der Maaten & Miller 1985a; Van Der Maaten ,Miller, & Whetstone, 1985b; Smith et al., 1990). Both intra-nasal and intra-muscular vaccines have been shown to enhance infectious bovine keratoconjunctivitis (George et al., 1988). Although attenuated live vaccines have many complicating side-effects associated with their use, they are currently offer the best protection against disease caused by bovine herpesvirus-1.

Alternatives to the attenuated live vaccines have been investigated. These alternatives consist of subunit or genetically altered virus with or without a unique adjuvant. Several preparations of subunit vaccines have been produced and tested by in

vivo challenge. These preparations have generally included the major glycoproteins gI, gIII, and gIV in combination with a variety of different adjuvants. There have been conflicting results as to the protective value these preparations have to offer. Efficacy against challenge was demonstrated for combinations of all the glycoproteins when administered with either ISCOMs or avridine as the immune adjuvant (Babiuk et al., 1987; Trudel et al., 1988). Separating the glycoproteins into individual vaccines was inconsistent as to its protective value. This may depend on the adjuvant used. When avridine was used the adjuvant, individual glycoproteins were capable of protecting against challenge (Babiuk et al., 1987). However, when Freund's incomplete adjuvant was used, individual glycoproteins were not protective against challenge (Israel, Marshall, & Letchworth, 1988). The results of these studies would indicate that multiple antigens need to be introduced into subunit vaccines and the appropriate adjuvant needs to be used for these preparations to induce protective immunity.

Genetically altered viruses provide a variety of opportunities to modify and improve the vaccine strain. The ideal result of these manipulations would be attenuation, deletion of virulent factors, stability, and the maintenance of immunogenicity. The herpesvirus genome is well suited for genetic manipulations because of its large size and the identification of superfluous proteins in vitro which could serve as the target for recombination and as markers for recombinants (Clark, 1987; Esposito & Murphy, 1989; Roizman et al., 1984; Roizman & Jenkins, 1985).

Several genetically altered BHV-1 strains have been isolated. Alterations in the virus genome have centered upon the thymidine kinase gene and glycoprotein III of the virus. Each of these targets provides a marker for identification of the recombinant. In the case of thymidine kinase, the recombinants can also be selectively enriched by using the nature of their inactive enzyme which fails to incorporate toxic thymidine analogs. Thymidine kinase negative BHV-1 isolates have been shown to be attenuated by their failure to produce clinical disease when introduced into a host animal (Kit et al., 1985; Kit, Kit, & McConnell, 1986; Whetstone et al., 1990a). Despite their attenuation, thymidine kinase negative BHV-1 strains can still establish latency within the host (Kit et al., 1985; Whetstone et al., 1990b). The thymidine kinase gene has also been used as a site for the introduction of heterologous genes into the genome. Vesciular stomatitis virus glycoprotein G has been expressed in cells infected with a BHV-1 recombinant (Lawrence, Whitbeck, & Bello, 1990). The latest BHV-1 vector expresses the capsid protein from foot-and-mouth disease virus (Kit et al., 1991).

A second commonly used site for mutational recombination of BHV-1 is the viral glycoprotein III. Isolation of gIII-deletion mutants have shown the glycoprotein dispensable for viral growth in cell culture (Nelson et al., 1989; Liang, Babiuk, & Zamb, 1991b). These mutants have not been tested in vivo to evaluate their attenuation. Their in vitro characterization, however, indicates the gIII-minus strains are defective in their attachment and rely on secondary attachment mechanisms (Liang et al., 1991a). This observation can indirectly influence viral virulence by reducing the number of cells

infected per unit of virus innoculum. Other researchers have overcome the question of gIII-minus attenuation by combining a gIII-minus mutation and a thymidine kinase mutation (Kit & Kit, 1990). Heterologous genes can also be introduced into the gIII gene. A BHV-1 gIII-minus recombinant has been engineered which expresses the Beta-galactosidase gene in infected cells (Liang, Babiuk, & Zamb, 1991b).

CHAPTER 2

Truncated Derivatives of Bovine Herpesvirus-1 Glycoprotein III Inhibit Antigen and Mitogen Induced Lymphoproliferation.

INTRODUCTION

Viruses may suppress the immune response of their hosts by inactivation of lymphocytes and phagocytic cells, stimulation of suppressor cells, and induction of immuno-suppressive proteins (Horohov & Rouse, 1986). This attribute of viral infections is of special significance when considering the latency and reactivation of bovine herpersvirus and its contribution to the colonization of secondary bacterial invaders in the bovine respiratory disease complex. Bovine herpesvirus-1 is capable of modifying the immune response using a variety of subtle mechanisms (Babiuk, Lawman, & Greiebel, 1989). Both viable and UV-inactivated BHV-1 virions have been shown to have a suppressive effect in vitro (Carter et al., 1989; Hutchings et al., 1990a). The following experiments have examined an independently expressed BHV-1 protein, glycoprotein III, for its contribution to BHV-1 immunosuppression outside of the context of the virion particle.

Fitzpatrick et al. (Fitzpatrick, Babiuk, & Zamb, 1989) conducted comparisons of the predicted amino acid sequence of gIII and identified a 96 amino acid domain having significant homology to the MHC class II antigen constant domain. A monoclonal antibody to gIII also cross reacted with a cell-surface glycoprotein on macrophages, polymorphonuclear leucocytes, and platelets (Fitzpatrick et al., 1990a). Other herpesviruses also have sequence or antigenic homology to

significant proteins which could provide a pathogenic advantage. Epstein-Barr virus open reading frame BCRF1 has homologous regions to cytokine synthesis inhibition factor (Moore et al., 1990). Human cytomegalovirus UL18 gene has sequence homology to the MHC class I molecule (Beck & Barrell, 1988) and its translated product complexes Beta2 microglobulin (Browne et al., 1990). Possessing homology to host proteins provides a selective advantage to viruses by enabling them to escape recognition by the host or to disable the host's immune response.

To examine this possibility in BHV-1, the following experiments expressed full-copy gIII in a transient eukaryotic expression system and created truncated derivatives which delete subsequent portions of the MHC class II homologous region. Using these derivatives in an in vitro lymphoproliferation assay, the influence of the MHC homologous region of BHV-1 gIII on bulk cultures of bovine peripheral blood mononuclear cells has been investigated. The following data indicate that gIII contains both immunogenic and suppressorgenic domains which influence bovine lymphocyte proliferation.

MATERIALS AND METHODS

Viruses and Cell Culture

Georgia bovine kidney (GBK) cells, free of bovine viral diarrhea virus, were used for propagating and titering virus stocks. COS-7 cells, Vero cells transformed to constituitively express the Large T antigen of SV40, were used for transfections and transient expression. Cell cultures were tested for the presence of mycoplasma and found

to be free of contamination. Bovine peripheral blood mononuclear cells (PBMC) were isolated the day of the assay. Established cell lines were grown in Dulbecco's Modified Eagle's medium (DMEM) with sodium bicarbonate (Sigma Chemical Company, St. Louis, MO) supplemented with 20mM HEPES, 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml of streptomycin. PBMC's were cultured in RPMI 1640 (Sigma Chemical Company, St. Louis, MO) with sodium bicarbonate supplemented with 2 mM L-Glutamine, 0.05 mM 2-mercaptoethanol, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml of streptomycin.

The Cooper strain of BHV-1, from the American Type Culture Collection (ATCC), was propagated in GBK cells at a multiplicity of infection (MOI) of 0.01, and incubated at 37°C until the cytopathic effect reached 100%. The supernate was clarified of cell debris by low speed centrifugation and then layered over a 20% sucrose cushion, and centrifuged for 2 hours at 100,000 x g. The virions were resuspended in growth medium and titrated. The virus was diluted to a concentration of 1×10^7 plaque forming unit (PFU)/ml and UV-inactivated (Model R52G, UVP, Inc., San Gabriel, CA) for 2 minutes at a distance of 12 cm prior to addition to lymphocyte cultures.

Construction of a BHV-1 genomic DNA library and expression of gIII.

BHV-1 Cooper strain (provided by Dr. J. Storz) genomic DNA was isolated by phenol extraction and dialysis (Maniatis, Fritsch, & Sambrook, 1982). The fragment encoding the gIII gene was subcloned as a 5.5 kb Bam HI-Eco RI section into a modified pUC19 containing a Bgl II restriction site in the polylinker (constructed by

Dr. K. Kousoulas). This plasmid, p867, was then digested with either Sac I, Xho I, or Sma I, and any overhanging ends blunted with T4 DNA polymerase using standard methods (Maniatis, Fritsch, & Sambrook, 1982). An Spe I* linker (New England Biolabs, Inc., Beverly, MA), containing a stop codon in all three reading frames, was ligated into place at the blunt Sac I, Xho I, and Sma I. Gene constructs were then cloned into the p91023 eukaryotic plasmid expression vector (Wong et al., 1985) as Bam HI-Eco RI fragments to create plasmids p2356, p2355, and p2357 (Figure 2.1). Full copy gIII was also cloned into p91023 to create p2323. After ligation into p91023, an Xho I-Eco RI fragment from p2323, containing the Adeno Major Late Promoter (AMLP) and the gIII coding sequence, was then introduced into vector pJNL-I (Scholler, 1988) to produce p2345. All plasmid DNAs used in transfections were purified twice in cesium chloride gradients.

Expression of glycoprotein III.

Expression of gIII in COS-7 cells was initially determined by indirect immunofluorescence assays (IFA). COS-7 cells were grown on circular coverslips in 24-well plates (Costar Inc., Cambridge, MA) and transfected as described. After 40 hours, the coverslips were fixed with methanol and blocked with 10% goat serum in PBS for 1 hour at room temperature. Monoclonal antibodies specific for gIII (supplied by Dr. Letchworth and Dr. Misra) were diluted 1:150 in PBS with 1% goat serum, and incubated with the monolayer for 1 hour at 37 C. The coverslips were washed in PBS and incubated with FITC

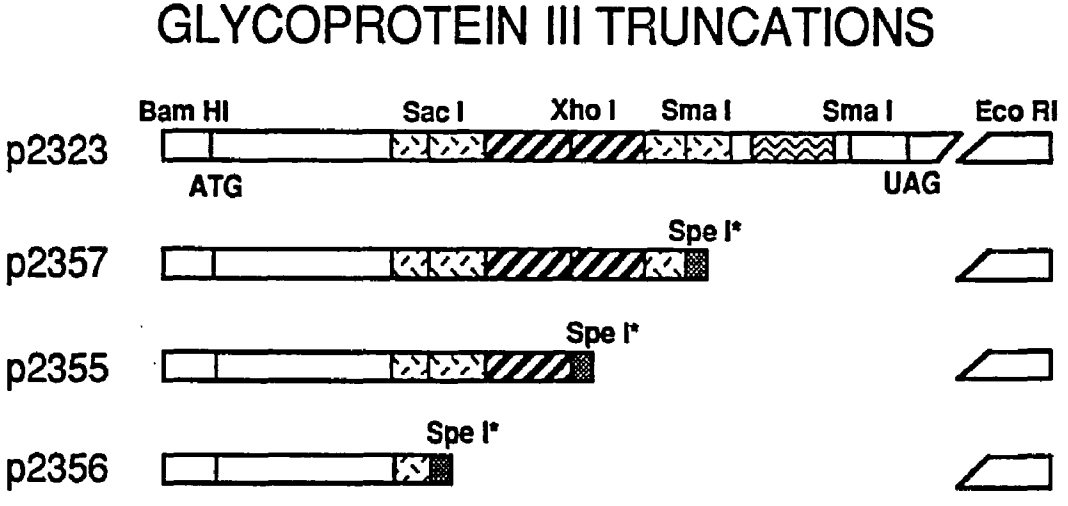

Figure 2.1

Construction of glycoprotein III truncations. The section of the genome containing the gIII gene was isolated as a Bam HI-Eco RI fragment. An Spe I* linker was ligated into the Sma I, Xho I, and Sac I sites of gIII, creating p2357, p2355, and p2356 respectively. is the intramembranous region. is the MHC class II homologous. are those regions showing homology to the Ig superfamily. (Fitzpatrick, Babiuk, & Zamb, 1989).

conjugated rabbit anti-mouse IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:50 and incubated for 1 hour at 37° C.

Radioimmunoprecipitation was carried out with cell-extracts obtained from transfected COS-7 cells largely according to a procedure outlined previously (Misra et al., 1982). Briefly, after incubating for 30 hours, transfected cells were washed with methionine-free media, and incubated 12 hours with 50 mCi of L- [^{35}S] methionine (New England Nuclear, Inc., Beverly, MA). Subsequently, cells were scraped and washed in PBS, RIPA buffer (0.05M TrisHCl pH=7.0, 0.15M NaCl, 1% DOC, 1% Triton X-100) was added and the cells sonicated for 4 seconds at a setting of 4 using a sonicator (Model 200, Branson, Danbury, CT). The cell debris was removed by centrifugation at 20,000 g for 30 minutes at 4° C. Monoclonal antibodies, at 5 ml per sample, were added to the clarified supernatant and then incubated on a rocking platform overnight at 4° C. Rabbit anti-mouse immunoglobulin G (Sigma Chemical Company, St Louis, MO) was added at 5 µl per sample and incubation continued for 4 hours. Protein A Sepharose CL-4B beads (Sigma Chemical Company, St. Louis, MO) were added at 10 mg per sample, and then incubated again for 1 hour at 4° C. The beads were then washed 5 times with RIPA buffer containing 0.1% SDS. Loading buffer (0.5M Tris pH=7.0, 3% sucrose, 2% SDS, 0.025% bromophenol blue was added and the samples were boiled for 5 minutes. Immunoprecipitated proteins were separated on a 9% denaturing polyacrylamide gel with a 3.5% stacking gel. Gels were treated with En^{3}Hance (New England Nuclear, Boston, MA) as directed, prior to autoradiography.

Transfection of COS-7 cells

COS-7 cells at 50% confluency in 25 cm^2 tissue culture flasks were transfected with 15 mg of plasmid DNA using a modified calcium phosphate precipitation technique (Graham & Van Der Eb, 1973). Plasmid DNA was mixed with 50 ml of 2.5M $CaCl_2$ and the volume brought up to 500 ul with distilled water. An equal volume of 2X HEPES-buffered saline (2XHBS: 280mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES acid, pH=7.05) was added to the DNA mixture with a constant bubbling of air. The solution was incubated at room temperature for 30 minutes. Flasks of COS-7 cells were washed once with tris-buffered saline (TBS: 137mM NaCl, 5mM KCl, 1.4mM Na_2HPO_4, 25mM Tris Base, 1.4mM $CaCl_2$, 0.5mM $MgCl_2$, pH=7.5) and incubated for 2 minutes with 0.5 mg/ml DEAE-dextran (Sigma Chemical Co., St. Louis MO) solution in TBS, after which the DNA mixture was added. Following a 4 hour incubation at 37°C, the cells were shocked with a 15% glycerol in 1X HBS for 2 minutes and washed with 1X HBS. Fresh media was added and the cells incubated for 48 hours. The supernate was collected, centrifuged to remove cell debris, and then aliquoted for storage at -20°C until used. Various volumes of the supernates (200, 70, 20, and 2 µl) were added to the lymphocyte cultures for testing.

Lymphoproliferation assay

Bovine peripheral blood mononuclear cells were obtained from three BHV-1 positive cattle (NO117, NO121, and NO130) and one BHV-1 negative cow (VMP001). The positive cattle all tested strong positive for BHV-1 by IFA at a serum dilution of 1:16. Venous blood

was collected into sterile tubes containing citric acid as an anti-coagulant. Buffy coat cells were obtained by centrifugation of the whole blood at 1000 x g for 45 minutes. PBMC were isolated by differential sedimentation of the buffy coat cells through a ficoll-hypaque gradient. The PBMC were washed twice with phosphate-buffered saline (PBS) and resuspended in growth medium at a final concentration of 1 X 10^6 cells/ml. Two mls of the cell suspension were added to each well of a 24-well plate along with various test supernates, viral suspensions, or Con A. All cultures were incubated at 39°C in a humidified, CO_2 incubator. At 48 hour intervals, the cultures were resuspended and 100 µl aliquots transferred to a 96-well plate for a 4 hour pulse with 0.5 µC of ^{3}H-thymidine. The radiolabelled cells were harvested onto glass fiber filter mats and counted in a liquid scintillation counter. The results are reported as net counts per minute (CPM) determined as the triplicate mean of the media control subtracted from the triplicate mean of the sample.

RESULTS

Expression of glycoprotein III in transfected COS-7 cells

COS-7 cells transfected with both p2323 and p2345 expressed full-copy gIII which reacted with gIII-specific MAbs by IFA (Figure 2.2A). High levels of gIII expression were detected by IFA in approximately 10% of the cells using different MAbs (Marshall, Israel, & Letchworth, 1988; Nelson et al., 1989). Expression of full-copy gIII expressed by p2323 and p2345 could also be detected by radioimmuno-precipitation followed by SDS-PAGE. Full-copy gIII expressed in

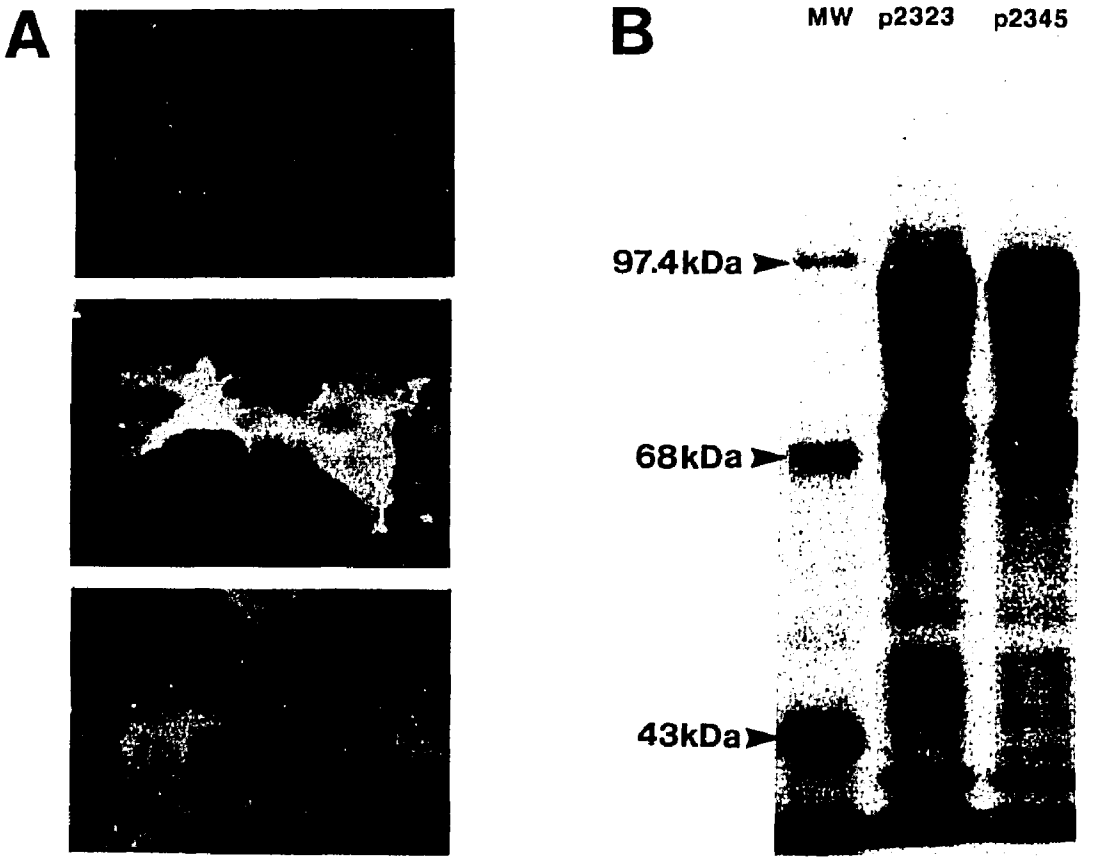

Figure 2.2

Expression of gIII by COS-7 cells. A) Immunofluorescence of COS-7 cells transfected with p91023 (top), p2345 (middle), p2323(bottom). B) Radioimmunoprecipitation of transfected COS-7 cells. Lane 1: ^{14}C molecular weight marker. Lane 2: COS-7 cells transfected with p2323. Lane 3: COS-7 cells transfected with p2345. Glycoprotein III appears as a band migrating at approximately 91 kDa.

COS-7 cells migrated in SDS-PAGE with a mobility consistent to the native-gIII (approximately 91kDa) specified by BHV-1 (Figure 2.2B). Radioimmunoprecipitations performed on the supernates of COS-7 cells transfected with p2323 and p2345 failed to detect gIII. Similarly, IFA and immunoprecipitations performed on supernates and cell extracts of COS-7 cells transfected with p2355, p2356, and 2357 which specify truncated versions of gIII failed to detect gIII-specific expression (results not shown).

Response to UV-inactivated BHV-1

Three BHV-1 seropositive and one seronegative cow were tested for their lymphoproliferative responses to UV-inactivated BHV-1. Lymphocytes from BHV-1 immune cattle proliferated in an antigen-specific (Figure 2.3A) and dose-dependent (Figure 2.3B) manner peaking on day 6. By contrast, lymphocytes obtained from the BHV-1 naive cow did not proliferate in response to UV-inactivated virions.

Response to gIII versions expressed in COS-7 cells.

Lymphocyte cultures from immune cattle responded to both full-copy and truncated derivatives of gIII expressed in COS-7 cells (Figure 2.4). Full-copy versions expressed by p2323 and p2345 stimulated approximately equal lymphocyte proliferation. Supernates from COS-7 cells transfected with p2357, p2355, and p2356 which contained truncated gIII-derivatives resulted in progressively increased levels of proliferation above the levels produced by the full-copy gIII alone. Lymphocytes from the naive cow did not proliferate in response to any of the gIII-derivatives expressed by COS-7 cells.

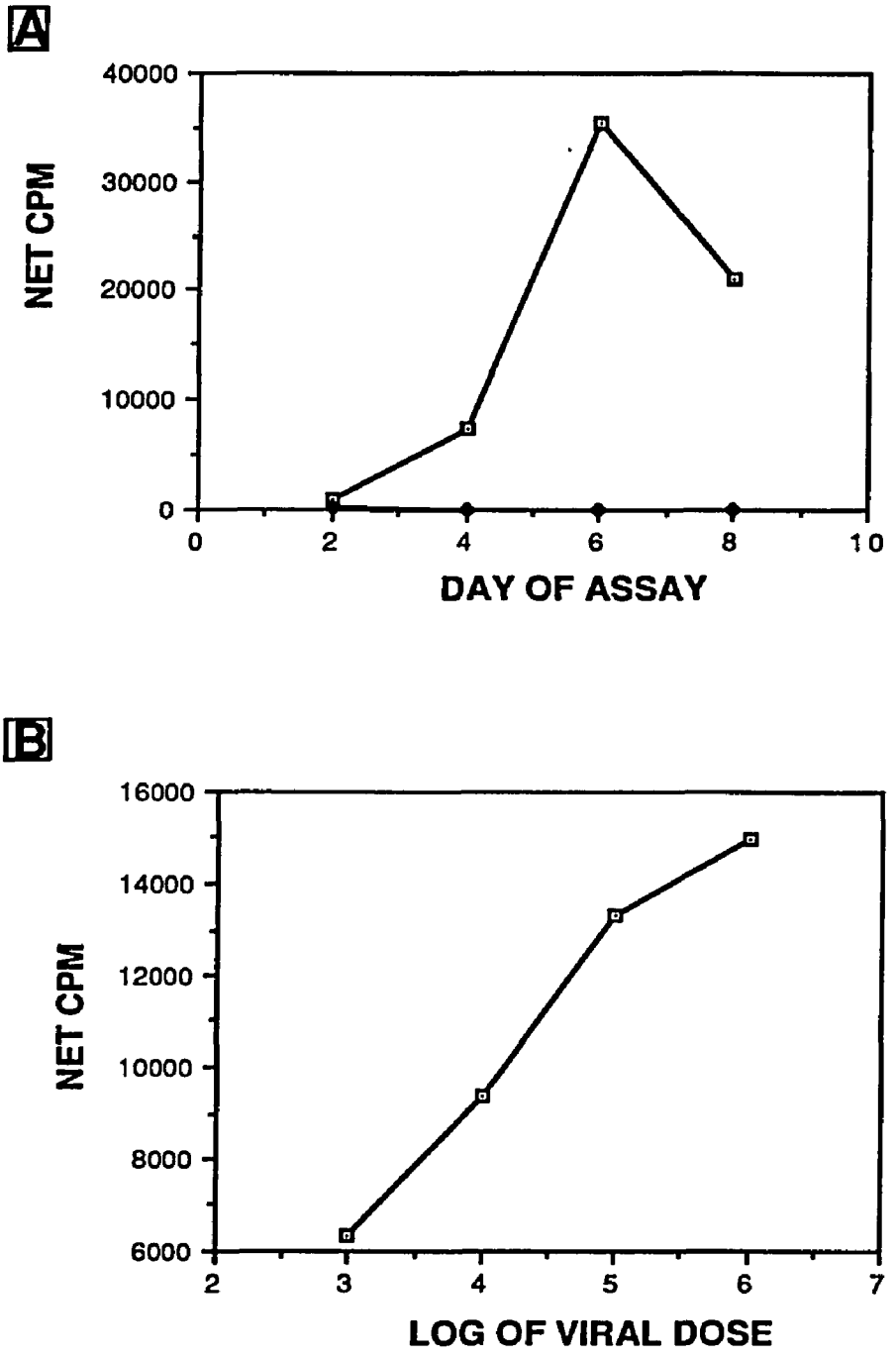

Figure 2.3

Lymphoproliferation to BHV-1. A) Response of immune and naive cows to 10^6 PFU's of UV-irradiated BHV-1 (Cooper Strain). B) Response of immune cow to increasing log doses of UV-irradiated BHV-1.

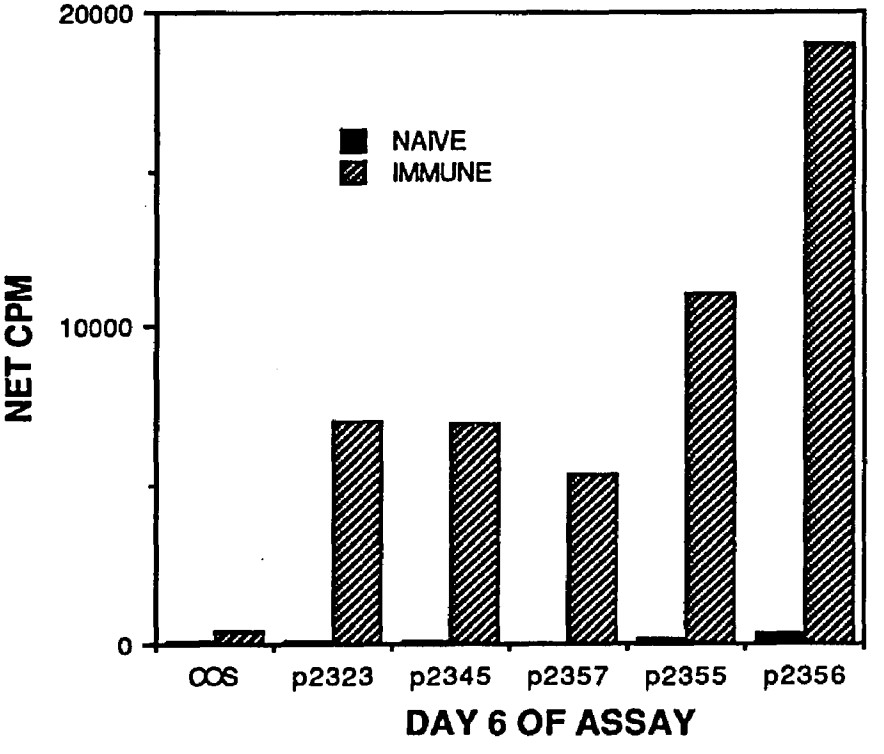

Figure 2.4

Day 6 proliferative response of immune and naive PBMC's to supernates. Supernates, at 200 µl, include mock-transfected COS-7 cells, full-copy gIII expressed by p91023 (p2323) and pJNL-1 (p2345), and truncated derivatives (p2357, p2355, p2356).

Responses to antigen in the presence of gIII derivatives

Since truncated versions of gIII appeared more effective at stimulating proliferation, we examined the effect of p2356 and p2323 supernates together compared with the effect obtained when an equal amount of supernate (200 µl) was used alone (Figure 2.5). Lymphocytes from all three immune cows responded to p2356-supernates at levels above the proliferation level when p2323 was used alone. p2323-supernates added to cultures containing p2356 caused a reduction in proliferation to an intermediate level between those obtained for p2356 and p2323-superates with lymphocytes from cow NO117. Addition of p2323 to P2356 cultures dropped proliferation to p2323 levels when lympocytes from cow NO121 were used. The most significant reduction in proliferation in response to the addition of p2323-superates were observed with lymphocytes from cow N0130 which exhibitted lower proliferation than p2323-superates alone.

The response to UV-inactivated BHV-1 was also affected by the presence of gIII-derivatives in the cultures (Figure 2.6). While lymphocytes from each of the immune cattle proliferated in response to 10^4 PFU of UV-inactivated BHV-1, a significant ($p<0.05$) drop in proliferation was noted when either p2356 or p2323 was added to cultures. p2323-supernates reduced proliferation of BHV-1 stimulated cultures more than p2356-supernates in lymphocyte cultures obtained from all three BHV-1 immune cattle.

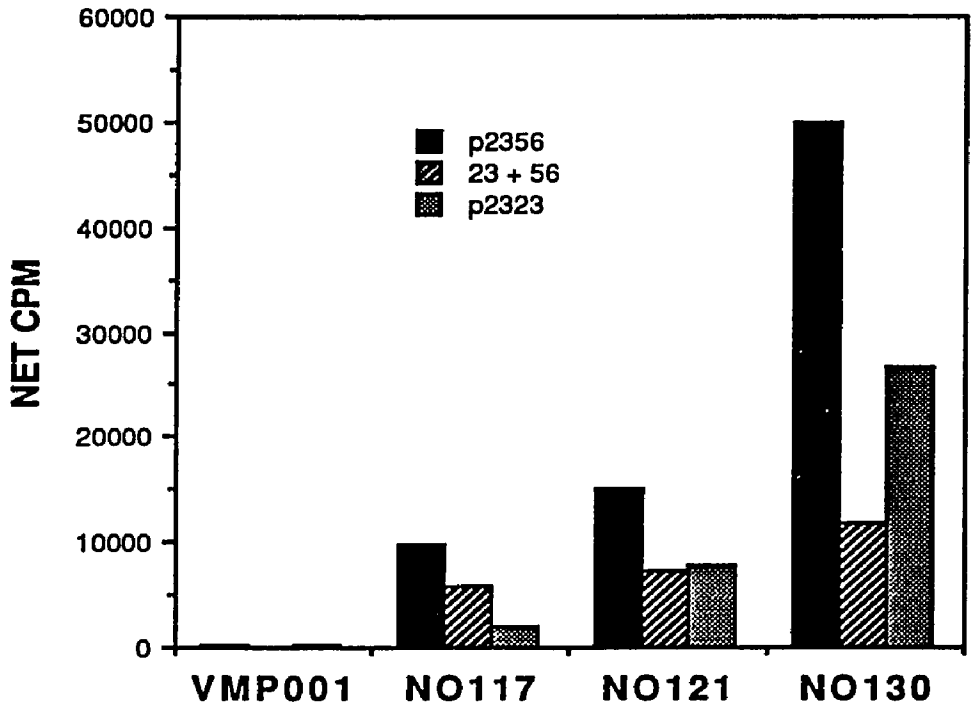

Figure 2.5

Effect of 200 µl of each supernate of full-copy gIII (p2323) on the proliferation to truncated derivative p2356. Day 6 proliferation responses are shown by naive cow, VMP001, and immune cows NO117, NO121, and NO130.

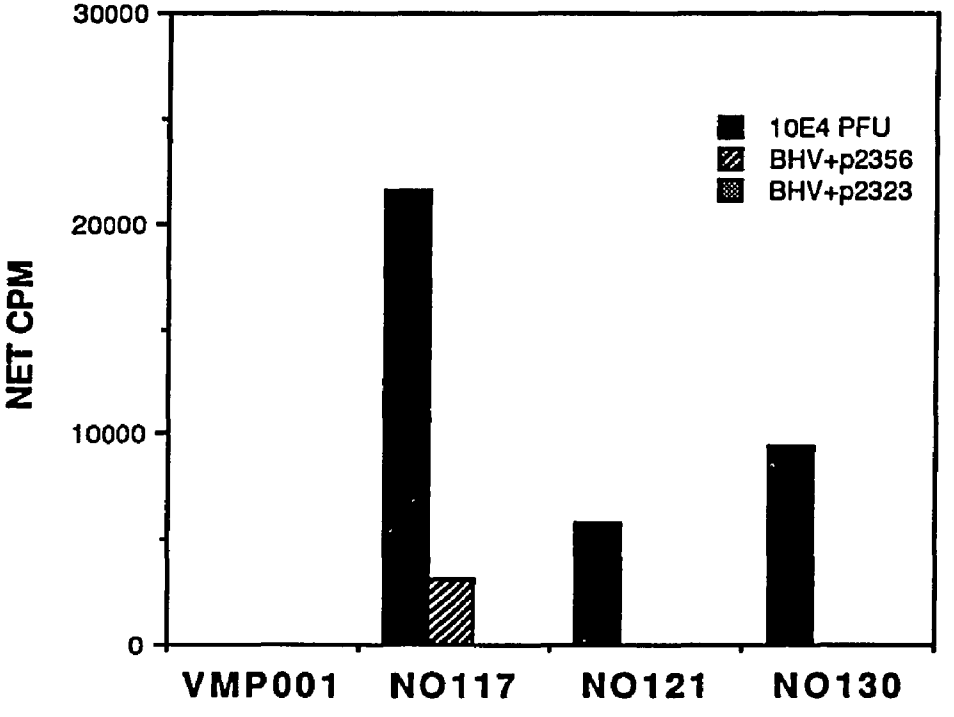

Figure 2.6

Effect of 200 µl of truncated and full-copy gIII derivatives on proliferation to 10^4 PFU of UV-irradiated BHV-1. Values represent day 6 proliferation results of naive (VMP001) and immune cattle (NO117, NO121, NO130).

Mitogenic responses in the presence of gIII derivatives.

To determine if the suppressive effect of gIII was virus specific, the effect of gIII-derivatives on the mitogenic responses to ConA was examined (Figure 2.7). In the presence of 2.5 mg of Con A and 200 µl of p2323, a statisticly significant ($p<0.05$) decrease in the mitogenic response of cows VMP001, NO121, and NO130 was observed in comparison to ConA alone, COS-7 cell supernate alone, and cultures incubated with supernates of COS-7 cells transfected with p2356. Cultures containing 200 µl of p2356-transfected COS-7 cells supernates did not exhibit similar statistics. Reducing the concentration of p2323-supernates from 70 µl to 20 µl restored the mitogenic responses to ConA.

DISCUSSION

Immunization studies and studies using purified glycoproteins in lymphoproliferation assays showed that cattle recognized gIII (Hutchings, Van Drunen Littel-Van Den Hurk , & Babiuk, 1990; Van Drunen Littel-Van Den Hurk, Gifford, & Babiuk, 1990a). However, gIII resulted in low levels of lymphocyte proliferation, and was the least protective against in vivo challenges. In this report, evidence is presented that BHV-1 gIII may contain both immunostimulatory and immunosuppressorgenic domains, and that the MHC-II-like domain of gIII may be part of the suppressorgenic domain

Expression off full-copy gIII by p2323 and p2345 was detected in COS-7 cell extracts by immunoprecipitation and IFA. However, full-copy gIII and gIII-truncated derivatives could not be detected by

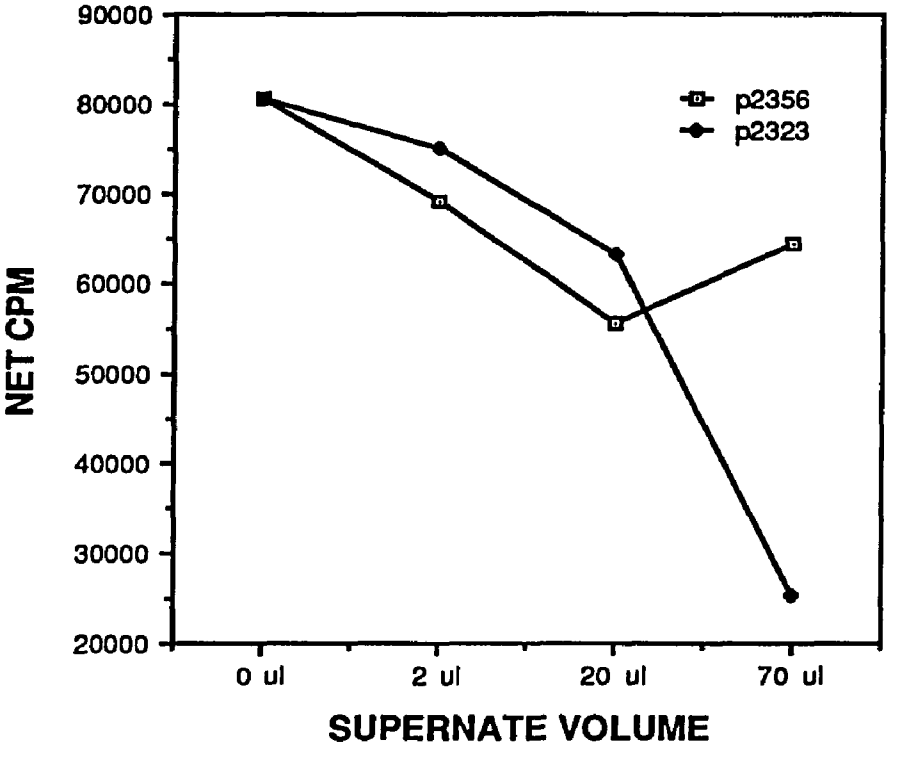

Figure 2.7

Inhibition of mitogenic response by increasing the concentration of p2323 supernate. Values represent mean of day 2 values.

immunoprecipitation in COS-7 cell supernates. Our failure to detect full-copy gIII and gIII-derivatives in the supernates of transfected cells was probably due to a combination of low amounts of gIII produced in COS-7 cells, low amounts of gIII secreted out of the COS-7 cells, and altered antigenicity caused by the truncations. While the secreted gIII peptides were not recognized by antibodies, they did stimulate an antigen-specific lymphoblast response.

It has been suggested that the MHC molecule can bind partially degraded antigen (Babbit et al., 1985; Gammon et al., 1987; Ametani et al., 1989). Those peptide fragments containing T-cell epitopes can stimulate both class I and class II restricted responses. (Townsend & Bodmer, 1989). Since the lymphoproliferative response to the COS-7 cell supernates were restricted to the BHV-1 immune cattle, it seems likely that the supernates contain T-cell epitopes. As the smallest constructs proved to be the the most stimulatory, it appears that the T-cell epitope is largely on the amino terminus. However, this differential proliferation could be attributed to the presence of a hindering structure within a partially processed protein (Brett, Cease, & Berzofsky, 1988), or to the revelation of a previously cryptic epitope (Ametani et al., 1989), Carboxy-terminal truncations of gIII may alter the conformation of gIII and cause the appearance of new epitopes that would normally be unavailable for MHC binding.

Alternatively, truncating gIII could result in the elimination of a suppressorgenic epitope (Sercarz & Krzych, 1991). Suppressive epitopes have been implicated in the nonresponsiveness of proteins as antigens. In vitro, this effect can be antigen specific by stimulating suppressor T-cells directed at the specific antigen target (Schwartz et

al., 1976; Sercarz et al., 1978; Krzych, Fowler, & Serçarz, 1985; Sercarz & Krzych, 1991). The presence of certain epitopes on a peptide may selectively stimulate T cells which can inhibit the response to the antigen. Thus in the same manner the elimination of a suppressor epitope enabled the response to lysozyme (Sercarz et al., 1978), elimination of the carboxy-terminus of gIII enabled bovine lymphocytes to respond to truncated gIII.

In addition, a general suppressive effect may also result by the action of the native protein on the immune response to the antigen, unrelated antigens, and non-specific mitogens (Copelan et al., 1983; Orosz et al., 1985; Viscidi et al., 1989). The presence of the MHC II homologous region in lymphocyte cultures significantly reduced the response to both mitogen and antigen (BHV-1 virions and gIII derivatives). While the mechanism of this suppressive effect is not known, the MHC class II homologous region may provide suppressive signals through the internalization of the peptide-MHC complex (Janeway, 1989). Just as the use of synthetic peptides has helped isolate the suppressive effect of other viral proteins (Cianciolo et al., 1985; Harrell et al., 1986; Chanh, Kennedy, & Kanda, 1988), similar experiments with synthetic peptides may help identify the mechanism of the suppressorgenic epitopes within gIII.

CHAPTER 3

Isolation and Characterization of a Glycoprotein III-Null Bovine Herpesvirus-1 Mutant

INTRODUCTION

Herpesvirus glycoproteins are involved in virus attachment, adsorption, penetration, and fusion to host cells (Cai, Gu, & Person, 1988; Campadelli-Fiume et al., 1988; Fuller, Santos, & Spear, 1989; Little et al., 1981; Herold et al., 1991). In addition, glycoproteins incorporated in the viral envelope and expressed on the surface of infected cells are targets of both humoral and cellular immunity.

Bovine herpesvirus-1 encodes for four distinct glycoproteins (Marshall, Rodriquez, & Letchworth, 1986; Misra, Blumenthal, & Babiuk, 1981). Three of these, gI, gIII, and gIV, are immunologically significant, being the targets of neutralizing antibodies and cell mediated immunity (Marshall, Israel, & Letchworth, 1988; Hutchings, Van Drunen Littel-Van Den Hurk, Hughes, & Babiuk, 1990b; Van Drunen Littel-Van Den Hurk, Gifford, & Babiuk, 1990a). The gene coding for glycoprotein III, a homolog to herpes simplex gC, has been mapped and sequenced, and shown to be nonessential for growth in cell culture (Fitzpatrick, Babiuk, & Zamb, 1989; Nelson et al., 1989; Liang et al., 1991a). Glycoprotein III is responsible for the hemagglutination of mouse red blood cells, and functions as an important viral protein for initial virus attachment to permissive cells (Trudel et al., 1987; Trepanier et al., 1985; Liang et al., 1991a; Okazaki et al., 1991). Its interaction with the cell is believed to be

mediated through surface heparan sulfate moieties (Okazaki et al., 1991).

KB3305 has been isolated as a gIII-null mutant of BHV-1 and used as a tool to investigate gIII's role in hemagglutination, attachment to permissive cells in the presence of heparin, and in the virus' ability to stimulate cellular immune responses tested in an in vitro lymphoproliferation assay.

MATERIALS AND METHODS

Virus and Cell Culture

Georgia bovine kidney (GBK) cells, free of bovine viral diarrhea virus, were used for propagating and titering virus stock. Rabbit skin cells were used for transfections and immunoperoxidase assay. Madin-Darby canine kidney cells were used for attachment studies to polarized cells. Cell cultures were tested for the presence of mycoplasma and found to be free of contamination. Bovine peripheral blood mononuclear cells (PBMC) were isolated the day of the assay. Established cell lines were grown in Dulbecco's Modified Eagle's medium (DMEM) with sodium bicarbonate (Sigma Chemical Company, St. Louis, MO) supplemented with 20mM HEPES, 5% fetal calf serum, 100 U/ml penicillin and 100 µg/ml of streptomycin. PBMC's were cultured in RPMI 1640 (Sigma Chemical Company, St. Louis, MO) with sodium bicarbonate supplemented with 2 mM L-Glutamine, 0.05 mM 2-mercaptoethanol, 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml of streptomycin.

The Cooper strain of BHV-1 (ATCC) was propagated in GBK cells at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C until

the cytopathic effect reached 100%. The supernate was clarified of cell debris by low speed centrifugation and then layered over a 20% sucrose cushion and centrifuged for 2 hours at 100,000 x g. The virions were resuspended in medium and titrated. The virus was diluted to a concentration of 1×10^7 PFU/ml and UV-inactivated (Model R52G, UVP, Inc., San Gabriel, CA) for 2 minutes at a distance of 12 cm prior to addition to lymphocyte cultures. Protein concentration of viral stock was determined by a Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA)

Isolation of gIII-null mutant virus

Bovine herpesvirus 1 viral DNA was isolated using the protocol outlined in Maniatis et al. for eukaryotic DNA isolation (Maniatis, Fritsch, & Sambrook, 1982). Rabbit skin cells at 50% confluency in 6-well tissue culture plates were cotransfected with 2 μg of viral DNA and 1 μg of plasmid using a modified calcium phosphate precipitation technique (Graham & Van Der Eb, 1973). Stocks of infectious virus were harvested from transfected rabbit skin cells after 4 to 7 days and screened for gIII-null phenotype using an immunoperoxidase assay on viral plaques. Infected monolayers were pretreated for 1 hour with 10% goat serum in serum free DMEM. The monolayers were incubated for 1 hour at 37° C with DMEM containing 1% goat serum and a 1:150 dilution of monoclonal antibodies to gIII, supplied by Dr. Letchworth and Dr. Misra. The cells were then washed twice with media and a 1:80 dilution of biotinylated horse antimouse-immunoglobulin G antibody in media was added and the plates incubated for 1 hour at 37° C. The cells were

washed with calcium-magnesium free PBS and incubated with the avidin: biotinylated horseradish peroxidase complex for 1 hour at room temperature. Finally the substrate, 10 mg of 4-chloro-1-napthol in 100 ml of CMF-PBS with 0.03% peroxide, was added and the color allowed to develop. Plaques were picked under agarose and purified four additional times.

Southern blot analysis

Hind III and Bam HI restriction enzyme digests of wild type and KB3305 were run on an 0.7% agarose gel overnight at 20V. The gels were stained, photographed, and the migration distance recorded with a UV-fluorescent ruler. The gel was depurinated by immersion in 0.30 N HCl for 5 minutes then denatured by immersion in 0.5M NaOH-1.5M NaCl for 10 minutes, followed by neutralization in 0.5M Tris-HCl, pH 7.4 and 3M NaCl for 15 minutes. This process was repeated a second time before the gel was transferred to a Nylon-1 membrane (GIBCO BRL, Gaithersburg, MD) for 45 minutes using a Trans-Vac TE80 vacuum blotter (Hoefer Scientific Instruments, San Francisco, CA. Transfer buffer, 25mM sodium phosphate pH 6.5, was added as needed over the blotting period. Following blotting, the gel was restained in ethidium bromide and evaluated for transfer. The membrane was baked for 2 hours at 80° C and then UV cross-linked for 4 seconds. Before hybridization, the membrane was incubated with 6X SSC, (1X SSC is 0.15M NaCl plus 0.15M sodium citrate), 1X Denhardt solution (0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.1% SDS, 0.2% bovine serum albumin), 30% formamide, and 10 μg/ml salmon

sperm DNA for 1 hour at 60° C in a hybridization incubator (Model 310, Robbins Scientific, Sunnyvale, CA).

The probe was prepared using the protocol included with the nick translation kit (NEK-004, DuPont, Boston, MA). Briefly, 0.5 µg of plasmid DNA were incubated with DNase 1 and DNA polymerase in the presence of ^{32}P labeled dCTP for 2 hours at 12° C. The volume was brought up to 200 µl and 5 µl of 20% SDS was added to stop the reaction. It was layered onto a G25 Sepharose column as described in Maniatis (Maniatis, Fritsch, & Sambrook, 1982) and spun to remove unincorporated label. The probe was kept on ice until ready to use and boiled for 5 minutes just prior to adding to the hybridization buffer.

The membrane was hybridized in 6X SSC, 1X Denhardt solution, and 100 µg/ml of salmon sperm DNA together with the probe. After hybridizing overnight at 60° C, the hybridization buffer was poured off and the membrane washed with 2X SSC. Prehybridization buffer without salmon sperm DNA was added and the membrane incubated for 60° C for 15 minutes. This process was repeated a second time or until the background radiation was reduced to an acceptable level. The membrane was then incubated at -70° C with Kodak X-Omat film using an intensifying screen.

Immunofluorescence and Immunoprecipitation

Monolayers of GBK cell were infected with wild type and KB3305 mutant at an MOI of 5, and allowed to incubate overnight. Infected cells were then spun onto glass slides using a cytospin centrifuge. Monoclonal antibodies specific for gIII (supplied by Dr. Letchworth

and Dr. Misra) were diluted 1:150 in PBS with 1% goat serum and incubated with the infected cells for 1 hour at 37° C. The coverslips were washed in PBS and incubated with FITC conjugated rabbit anti-mouse IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:50 and incubated for 1 hour at 37° C.

Radioimmunoprecipitation was carried out on infected cells using the procedure outlined by Misra (Misra et al, 1982). After incubating for 30 hours, transfected cells were washed with methionine-free media and incubated with 50 μCi of L-[^{35}S] methionine (New England Nuclear, Inc., Boston, MA) overnight. Cells were scraped and washed in PBS the following morning. RIPA buffer (0.05M TrisHCl pH=7.0, 0.15M NaCl, 1% DOC, 1% Triton X-100) was added and the cells sonicated for 4 seconds at a setting of 4 using a sonicator (Model 200, Branson, Danbury CT). The cell debris was removed by centrifugation at 20,000 g for 30 minutes at 4° C. Monoclonal antibodies, at 5 μl per sample, were added to the clarified supernatant and then incubate on a rocking platform overnight at 4° C. Rabbit anti-mouse immunoglobulin G (Sigma Chemical Company, St Louis, MO) was added at 5 ul per sample and incubation continued for 4 hours. Washed Protein A Sepharose CL-4B beads (Sigma Chemical Company, St. Louis, MO) were added at 10 mg per sample and then incubated again for 1 hour at 4° C. The beads were then washed 5 times with RIPA buffer containing 0.1% SDS. Loading buffer (0.5M Tris pH =7.0, 3% sucrose, 2% SDS, 0.025% bromophenol blue was added and the samples were boiled for 5 minutes. Immunoprecipitated proteins were separated on a 9% denaturing polyacrylamide gel with a 3.5% stacking gel. The gel was run

overnight at 60V on an Protean II electrophoresis rig (Bio-Rad Laboratories, Richmond, CA). The gel was stained for 30 minutes (45% methanol, 10% acetic acid 2.5% Coomassie blue), destained until the marker could be identified, rehydrated and exposed to En^{3}Hance (New England Nuclear, Boston MA) as directed. The gel was incubated with Kodak X-OMAT AR film at -70° C for 3-4 days.

Lymphoproliferation assay

Bovine peripheral blood mononuclear cells were obtained from three BHV-1 positive cattle (NO117, NO121, and NO130) and one BHV-1 negative cow (VMP001). The positive cattle tested as +4 positive for BHV-1 by IFA, at a serum dilution of 1:16. Venous blood was collected into sterile tubes containing citric acid as an anti-coagulant. Buffy coat cells were obtained by centrifugation of the whole blood at 1000 x g for 45 minutes. PBMC were isolated by differential sedimentation of the buffy coat cells through a ficoll-hypaque gradient. The PBMC were washed twice with phosphate-buffered saline (PBS) and resuspended in medium at a final concentration of 1 X 10^6 cells/ml. Two mls of the cell suspension and various viral dilutions were added to each well of a 24-well plate. All cultures were incubated at 39° C in a humidified, CO_2 incubator. At 48 hour intervals, the cultures were resuspended and 100 ul aliquots transferred to a 96-well plate for a 4 hour pulse with 0.5 μCi of ^{3}H-thymidine. The radiolabelled cells were harvested onto glass fiber filter mats and counted in a liquid scintillation counter. The results are reported as net counts per minute (CPM) which is

determined by the triplicate mean of the media control subtracted from the triplicate mean of the sample.

RESULTS

Isolation of BHV-1 gIII-null virus.

KB3305 was isolated as a gIII-null mutant arising from a cotransfection experiment in which naked viral DNA and a plasmid were transfected into low passage rabbit skin cells. Infectious viral plaques formed after cotransfection and were screened by the black plaque assay for gIII-null mutants using a pool of monoclonal antibodies. Figure 3.1 illustrates the distinction of white versus black plaques in rabbit skin cells. The gIII-null mutants constituted approximately 10% of the total virus population. KB3305 was picked based on its distinct plaque morphology shown in Figure 3.2. KB3305 was easily differentiated from wild type on the basis of plaque morphology. Its distinguishing feature was a honeycomb appearance to the center of the plaque compared to a vacant plaque center in the wild type and other mutants.

After extensive plaque purification, we tested the viral genome for the presence of possible large modifications in the gIII gene resulting from recombination of the plasmid with the viral genome. A β-galactosidase cassette was cloned within the gIII gene contained within the Hind III "I" fragment cloned into plasmid p2379. Figure 3.3 represents the results of a Southern blot using ^{32}P labeled p2379 as the probe. Both the Hind III and Bam HI fragments of KB3305 were identical to the wild type, indicating that a recombinational event which would have introduced the complete Bam HI fragment of

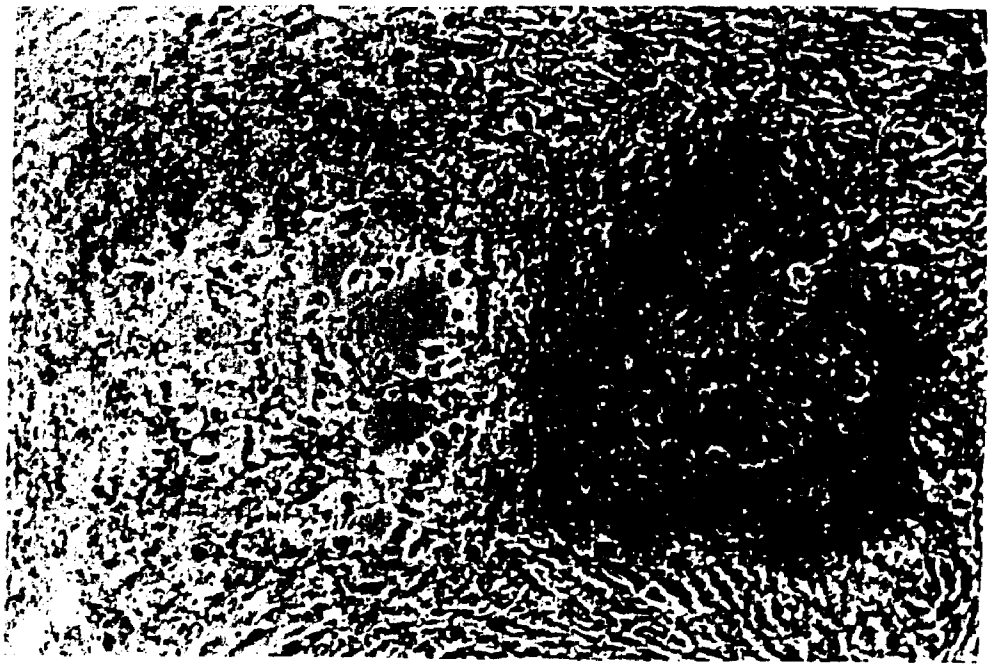

Figure 3.1

Immunoperoxidase staining of KB3305 and wild type (ATCC) BHV-1 plaques in rabbit skin cells. Wild type (ATCC) BHV-1 plaques precipitates substrate and appears black. KB3305 plaques do not precipitate substrate and appear "white" or colorless.

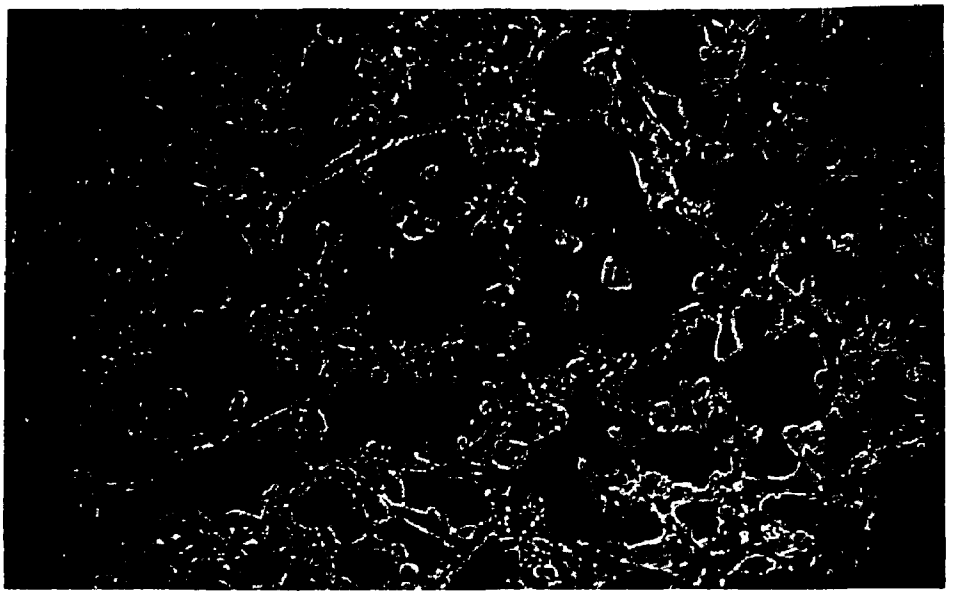

Figure 3.2
Morphology of KB3305 plaques in Georgia bovine kidney cells. The center of the plaque shows the characteristic honeycomb configuration of the mutant plaque.

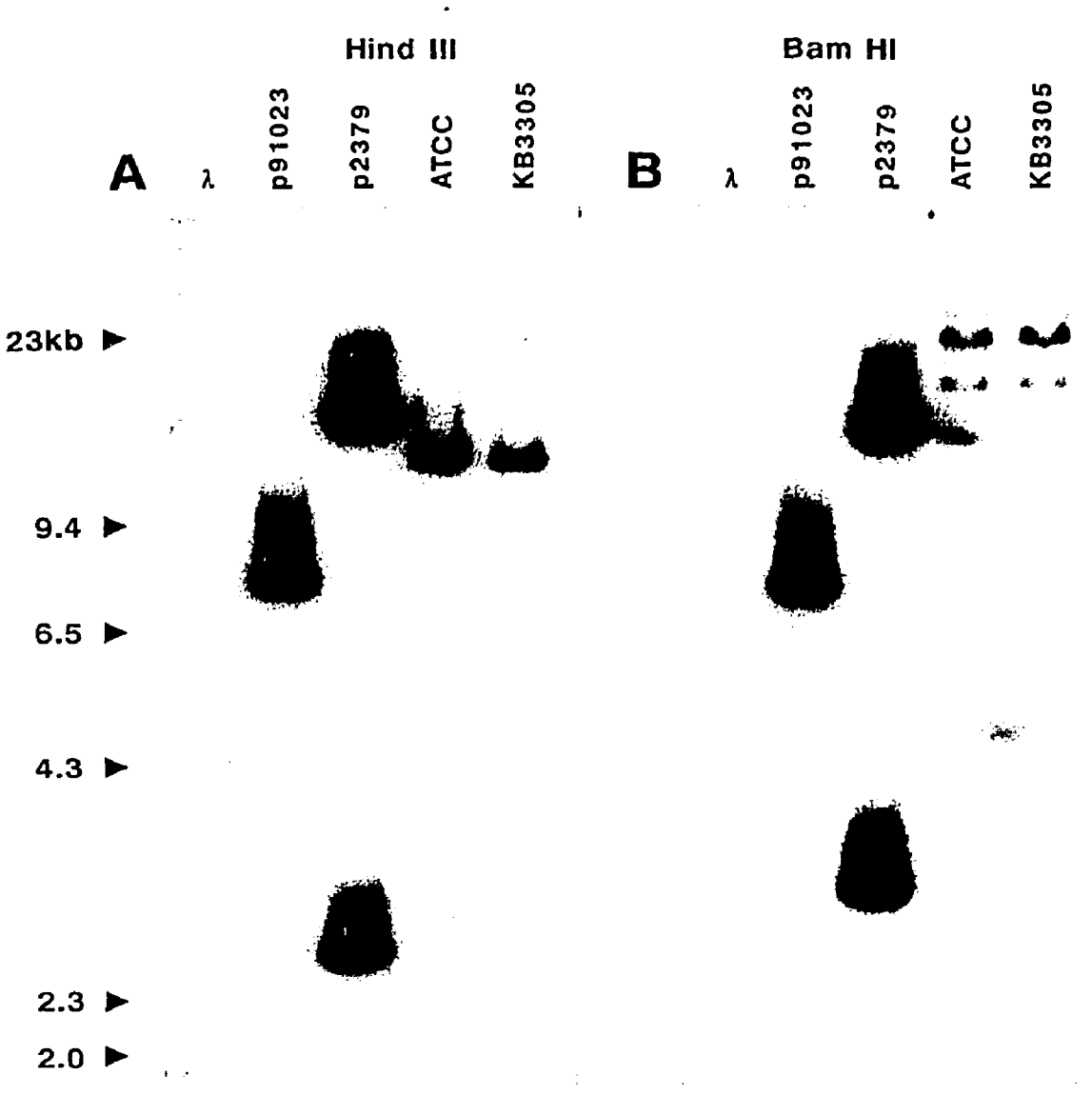

Figure 3.3

Southern blot comparing wild type (ATCC) BHV-1 and KB3305 hybridization to the recombinant plasmid p2379. Hind III digested lambda DNA served as a negative control. p91023 and p2379 acted as positive controls. The viral DNA was digested with either Hind III (A) or Bam HI (B). For the mutant to have recombined, the lower Bam HI fragment of p2379 would need to be present in the viral lanes.

p2379 into the genome did not occur. The appearance of identical DNA fragments in KB3305 and parent wild type strain indicated that there were no large modifications of the gIII gene, although small deletions or insertion or point mutations can not be excluded.

To confirm KB3305 was a gIII-null mutant, we assayed infected GBK monolayers by immunofluoresence and radioimmunoprecipitation. Using monoclonal antibodies to gI, gIII and gIV, cells infected with KB3305 reacted only when incubated with monoclonals to gI and gIV. A pool of monoclonals to gIII failed to react under the same conditions. Cells infected by wild type virus reacted with antibodies to all three glycoproteins. Radioimmunoprecipitation was carried out on ^{35}S labeled cells infected with wild type and KB3305 using monoclonal antibodies to gI, gIII, and tegument protein VP8. Figure 3.4 illustrates the comparison of KB3305 to wild type for expression of viral proteins. VP8 and gI were expressed by both wild type and KB3305. Glycoprotein III failed to precipitate from KB3305, but was clearly present in the wild type.

Hemagglutination assay

Hemagglutination was performed in 96-well round bottom plates. Twenty-five microliters of CMF-PBS were added to each well. Two-fold dilutions of wild type or KB3305 were serially diluted into the wells. Erythrocytes from adult BALB/C mice were collected in Alsever solution and washed three times in CMF-PBS. The mouse RBC suspension was diluted to 0.3% and 50 µl added to each well. The microtiter plate was left undisturbed for 2 hours at room temperature. The titer was expressed as the inverse of the dilution.

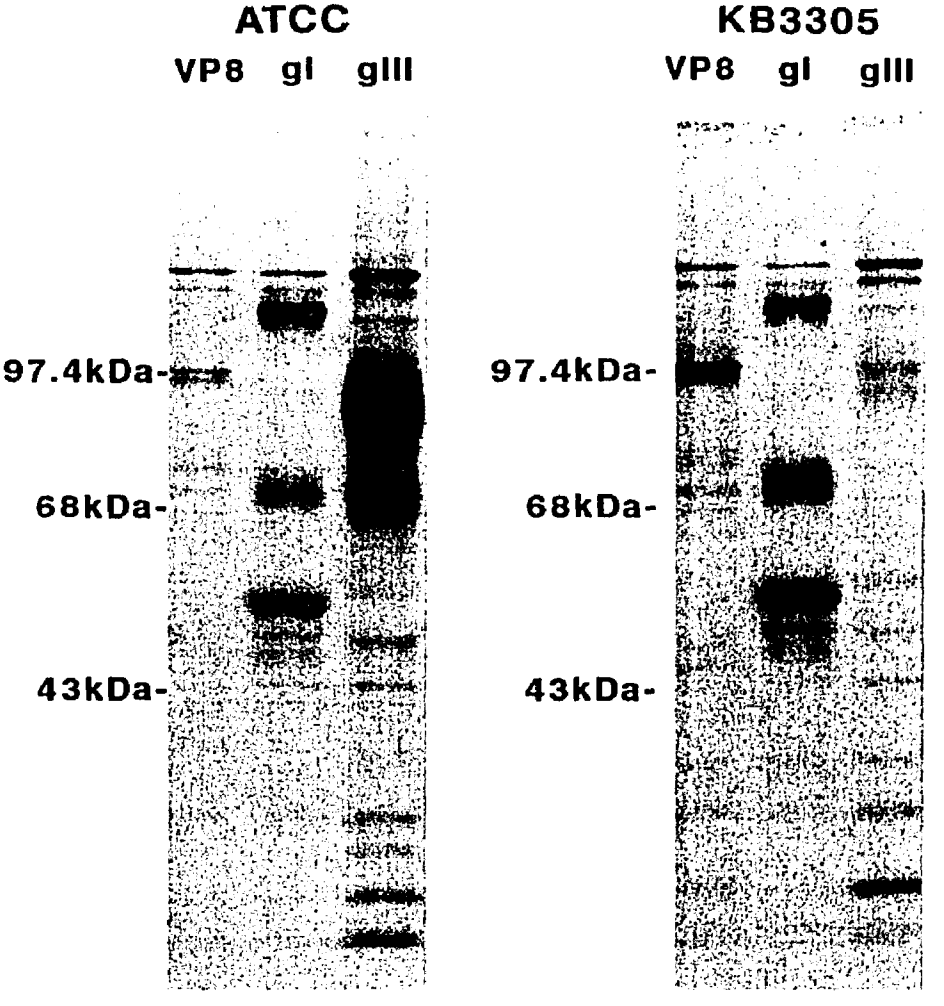

Figure 3.4

Radioimmunoprecipitation of ^{35}S-methionine labeled Georgia bovine kidney cells infected with wild type (ATCC) BHV-1 and KB3305. Monoclonals antibody to VP8, gI, and gIII were used to isolate the viral proteins.

Table 3.1 presents the hemagglutination results of wild type versus KB3305. Wild type virus hemagglutinated mouse red blood cells at concentrations of 2 X 10^6 and above. KB3305 failed to hemagglutinate mouse red blood cells at similar concentrations.

Growth characteristics of KB3305

GBK cell monolayers in 6 well plates were infected with either wild type or KB3305 virus at an MOI of 1. At 4 hour intervals, the wells were scraped and centrifuged to separate supernant from infected cells. The cells were frozen and thawed three times to release intracellular virions. Both cellular and supernant samples were then titered on GBK cells. The growth characteristics of KB3305 were compared to its wild type strain. This comparison is shown in Figure 3.5. Both viruses replicated equally well during the first 8 hours. The growth of cell-associated KB3305 lagged behind wild type by 10 fold after 24 hours. The wild type produced 15 fold more extracellular virions than did KB3305 at a level equivalent to its intracellular levels. KB3305 yielded 5 fold less extracellular virions when compared to its intracellular level.

To examine the effect of temperature on KB3305's growth, parallel infections of wild type and KB3305 were set up on GBK cell monolayers and incubated at 37° C and 39° C. Plaques were counted after 48 hours. The mutant grew at equivalent levels at 39° C as it did at 37° C.

Table 3.1
Hemagglutinating activity of wild type (ATCC) BHV-1 and KB3305. Erythrocytes from BALB/C mice were diluted to 0.3% and incubated with the above virus titers expressed as PFU's.

Titer	ATCC	KB3305
8×10^6	+	-
4×10^6	+	-
2×10^6	+	-
1×10^6	-	-
5×10^5	-	-

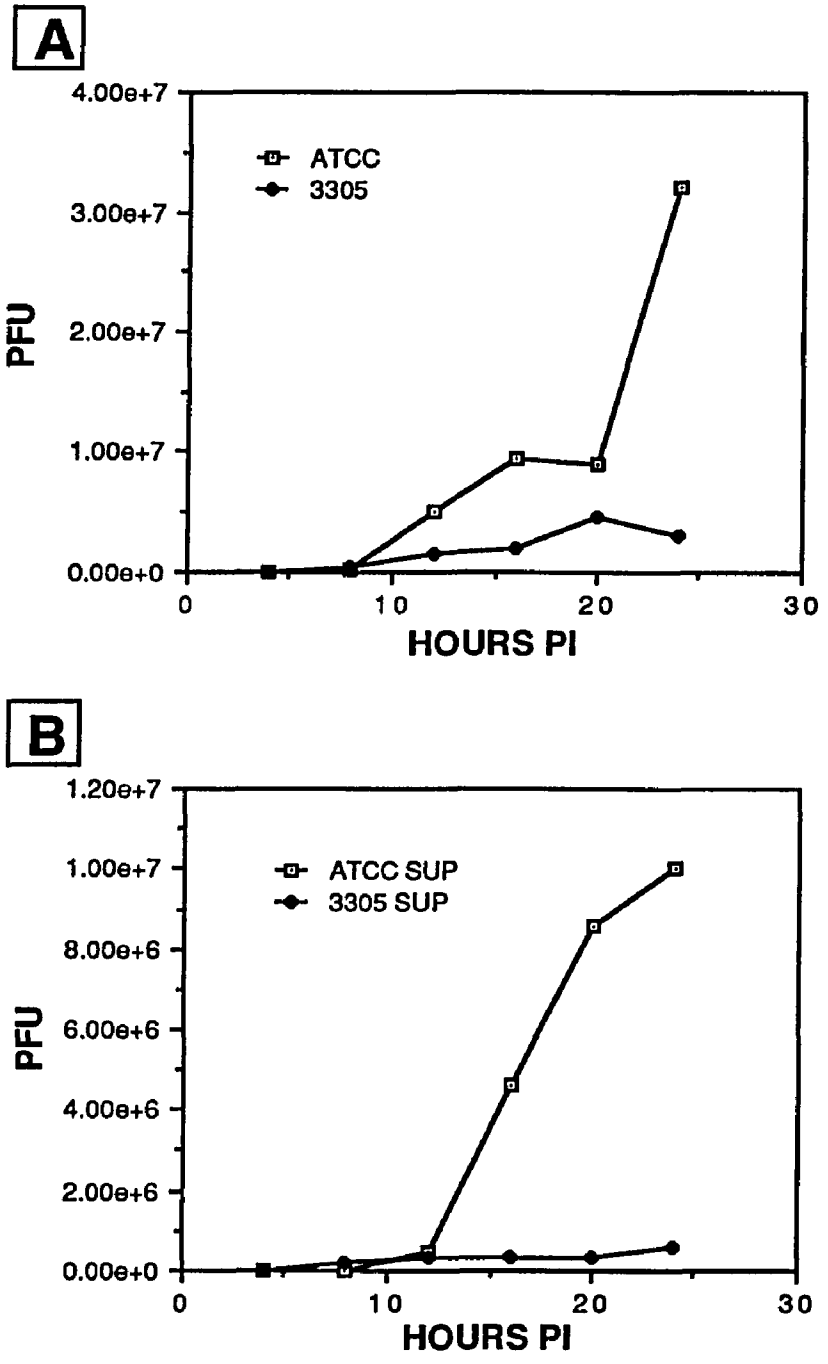

Figure 3.5

Single step growth curves comparing wild type (ATCC) BHV-1 and KB3305. (A) Graph of results of cell associated virus of wild type (ATCC) BHV-1 and KB3305. (B) Graph of results of extracellular virus of wild type (ATCC) BHV-1 and KB3305.

Virus adsorption in the presence of heparin

Heparin (Sigma Chemical Co., St. Louis, MO) was added to dilutions of wild type or KB3305 virus at a final concentration of 100 U/ml. GBK monolayers, at 90% confluency were incubated, with the virus-heparin solution for 1 hour at 37° C. Parallel cultures were also infected with virus which did not contain heparin for comparison of plaque forming capabilities. The infection broth was aspirated off and a 1% methylcellolose overlay in DMEM was added to the cells. Plaques were counted after the plates were incubated for 48 hours at 37° C. Figure 3.6 illustrates the effect of heparin when added to the infection broth. The presence of heparin reduced the wild type titer by 75% compared to those cultures without heparin. In the case of KB3305, the titer was reduced by 65%.

Lymphoproliferation to gIII-null mutant

In order to establish the antigenic potential of KB3305, the in vitro lymphoproliferative response of bovine peripheral blood mononuclear cells to the mutant were compared to that of the wild type. Figure 3.7 shows the typical response of immune cattle to both viruses. PBMC's from BHV-1 immune cattle exhibited similar proliferative responses to bothe the wild type virus and KB3305 despite the later's lack of one of its major anitgens.

DISCUSSION

The gIII-null BHV-1, KB3305, has been used as a tool to investigate the role of gIII in the infection of permissive cells and on the in vitro immune response. Mutant KB3305 produces a distinct

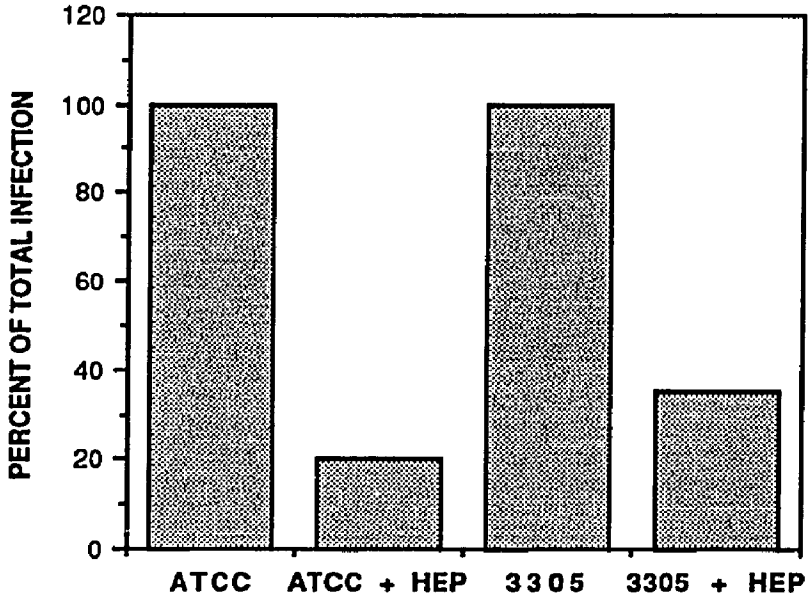

Figure 3.6

Graph of heparin sensitivity of wild type (ATCC) BHV-1 compared to KB3305. GBK cell monolayers were infected with wild type and KB3305 BHV-1 in the presence of 100 U/ml of heparin and without heparin.

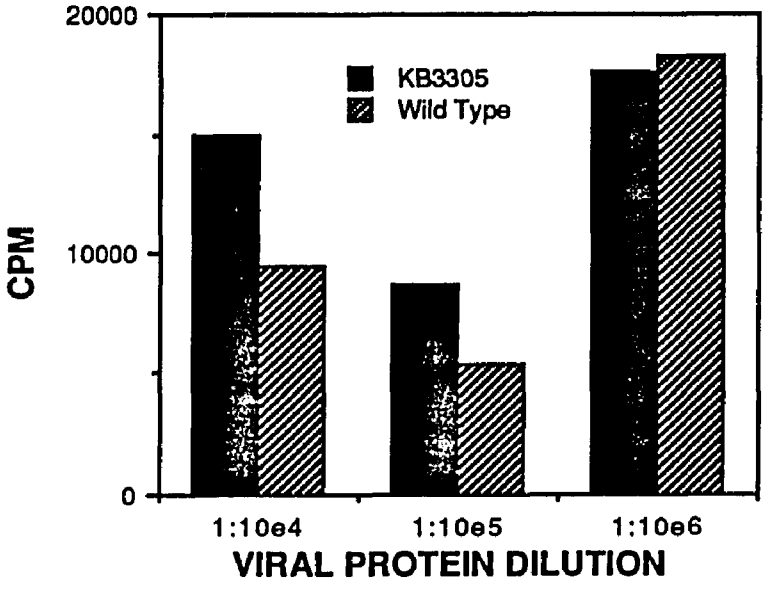

Figure 3. 7

Graph of lymphoproliferative response a BHV-1 immune cow to equal protein concentrations of KB3305 and wild type (ATCC) BHV-1.

plaque morphology, is more cell associated and shows a reduced growth rate in cell culture when compared to the wild type. Previous research on a different gIII-null mutant indicated that the reduced growth rate associated with the virus was caused by defective adsorption rather than faulty replication (Liang et al., 1991a). KB3305 may still be able to replicate efficiently in cells, but its release from the cell or its interference with host processes may be modified as indicated by its less severe cytopathology.

The hemagglutinating phenomenon of BHV-1 is limited to mouse red blood cells and varies between mouse strains (Trepanier et al., 1985). Trudel et al. identified the hemagglutinating component of BHV-1 to be gIII using monoclonal antibodies to inhibit hemagglutination (Trudel et al., 1987). KB3305 failed to hemagglutinate mouse red blood cells in comparison to hemagglutinating concentrations of the wild type.

Herpesviruses have been shown to initially bind heparan sulfate on the surface of cells (WuDunn & Spear, 1989; Mettenleiter et al., 1990; Okazaki et al., 1991). Using affinity chromatography, Okazaki (Okazaki et al., 1991) showed that only gIII of BHV-1 bound to heparin-agarose columns. KB3305 was sensitive to adsorption inhibition by heparin. Both gC and gB of herpes simplex have been found to bind heparin-Sepharose affinity columns (Herold et al., 1991). In the case of pseudorabies virus, its gIII-a gC homolog, and gII-a gB homolog were found to bind to heparin-Sepharose columns (Mettenleiter et al., 1990). However, pseudorabies virus gII depended on the presence of gIII for effective binding. Heparin may be binding

to BHV-1 gI, the HSV-1 gB homolog, which in the absence of gIII may serve as a secondary viral attachment protein.

Previous work on truncated gIII derivatives indicated that this glycoprotein contained immunosuppressive epitopes within its MHC class II homologous region (Byrne, Horohov, & Kousoulas, 1992). Therefore, the proliferation of bovine lymphocytes to KB3305 was examined and compared with the wild type. These results indicate immune cattle recognized gIII-null virions as wild type. It did not appear that deleting gIII from the virus provided any advantage to the immune response, even though the virus lacked the regions previously identified as immunosupppressive. The role of the immunossupressive domain on gIII in virus-induced immunossuppression remains unclear.

SUMMARY

Modified live viruses have historically been used to protect against viral diseases. With the application of molecular biology, techniques for the production of vaccine strains have included genetic recombinants with designed deletions or insertions into the original viral genome. These alterations can attenuate and genetically mark the recombinant strain.

This research has investigated the role of bovine herpesvirus-1 glycoprotein III in the pathogenesis and immunosuppression exhibited during BHV-1 infections. By independently expressing gIII and creating carboxy-terminus truncations, the MHC class II homologous region of gIII has been shown to have an inhibitory effect on the proliferation of bovine lymphocytes to antigen and mitogen. This observation indicates an advantage to producing gIII-null mutants. These mutants would not only lack an immunosuppressive protein, but would also be readily identifiable by their gIII-null marker.

A gIII-null virus, KB3305, was isolated as a means to examine the importance of gIII to viral infections, and to determine any advantages a gIII-null virus would provide to the immune response. In the process of characterizing the virus, the role of gIII was found to be important to the attachment and growth of the virus in cell culture. The data from heparin sensitivity studies indicated other proteins were also involved in the initial attachment of the virus to the cells. The function of gIII as the sole hemagglutinating protein was clarified by the inability of KB3305 to agglutinate red blood cells.

Finally, KB3305 was able to stimulate bovine lymphocytes in an equivalent manner to wild type virus, indicating deletion of a major immunologic target of BHV-1 does not hinder the cellular immune response.

REFERENCES

AMETANI A., APPLE R., BHARDWAJ V., GAMMON G., MILLER A., SERCARZ E. (1989) Examining the crypticity of antigenic determinants. *CSH Symp. Quant. Biol.* **54**:505-511.

BABBITT B.P., ALLEN P.M., MATSUEDA G., HABER E., UNANUE E.R. (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature.* **317**:359-361.

BABIUK L.A., L'ITALIEN J., VAN DRUNEN LITTEL-VAN DEN HURK S., ZAMB T., LAWMAN J.J.P., HUGHES G., GIFFORD G.A. (1987) Protection of cattle from bovine herpesvirus type 1 (BHV-1) infection by immunization with individual viral glycoproteins. *Virology.* **159**:57-66.

BABIUK L.A., LAWMAN J.J.P., GREIEBEL P. (1989) Immunosuppression by bovine herpesvirus 1 and other selected herpesviruses. In: Virus-Induced Immunosuppression. S. Specter, M. Bendinelli, H. Friedman eds. Plenum Press, New York NY. pp.141-171.

BABIUK L.A., OHMANN H.B. (1985) Bovine herpesvirus-1 (BHV-1) infection in cattle as a model for viral induced immunosuppression. In: Viral Mechanisms of Immunosuppression. M. Kende, J. Gainer, H. Chirigos, eds. Alan R. Liss, Inc. New York NY. pp.99-114.

BAKER J.C., RUST S.R., WALKER R.D. (1989) Transmission of a vaccinal strain of infectious bovine rhinotracheitis virus from intransally vaccinated steers commingled with nonvaccinated steers. *Am. J. Vet. Res.* **50**:814-816.

BANDYOPADHYAY S.K., MITTAL S.K., FIELD H.J. (1990) Identification of the gene homologous to HSV major DNA binding protein in the BHV-1 genome. *Vet. Microb.* **22**:203-212.

BARANOWSKI E., DUBUISSON J., PASOTRET P., THIRY E. (1991) Identification and characterization of new bovine herpesvirus-1 glycoproteins by using monoclonal antibodies. 10B-18 p.249 Abstr. 16th International Herpesvirus Workshop. Pacific Grove, California.

BECK S., BARRELL B.G. (1988) Human cytomegalovirus encodes a glycoprotein homologous to MHC class-1 antigens. *Nature*. **331**:269-272.

BIELANSKI A., LOEWEN K.G., HARE W.C.D. (1988) Inactivation of bovine herpesvirus-1 (BHV-1) from in vitro infected bovine semen. *Theriogenology*. **30**:649-657.

BLEWETT E.L., MISRA V. (1990) The proteolytic cleavage of bovine herpesvirus-1 glycoprotein B is not essential for its function. 248 p.45 Abstr. 71st Ann. Mtg. Conf. Res. Workers Anm. Dis. Chicago, IL.

BLOOD D.C., TADOSTITS O.M. eds. (1989) Veterinary Medicine. 7th edition. Bailliere Tindall W.B. Saunders. Philadelphia PA. pp. 899-906.

BRAKE F., STUDDERT M.J. (1985) Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo and caprine herpesviruses 1 and bovine encephalitis herpesvirus. *Austr. Vet. J.* **62**:331-334.

BRETT S.J., CEASE K.B., BERZOFSKY J.A. (1988) Influences of antigen processing on the expression of the T cell repertoire: Evidence for MHC-specific hindering structures on the products of processing. *J. Exp. Med.* **168**:357-373.

BROWNE H., SMITH G., BECK S., MINSON T. (1990) A complex between the MHC class I homologue encoded by human cytomegalovirus and ß2 microglobulin. *Nature*. **347**:770-772.

BYRNE K.M., HOROHOV D.W., KOUSOULAS K.G. (1992) Truncated derivatives of bovine herpes virus-1 glycoprotein III inhibit anitgen and mitogen induced lymphoproliferation. Submitted *J. Virol.* 1992.

CAI W.H., GU B., PERSON S. (1988) Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* **62**:2596-2604.

CAMPADELLI-FIUME G., AVITABILE E., FINI S., STIRPE D., ARSENAKIS M., ROIZMAN B. (1988) Herpes simplex virus glycoprotein D is sufficient to induce spontaneous pH-independent fusion on a cell line that constitutively expresses the glycoprotein. *Virology* . **166**:598-602.

CARPENTER D., MISRA V. (1990) BHV-1 homologue to HSV-1 UL47, a regulator of the alpha-trans inducing factor. p.54 Abstr. 15th International Herpesvirus Workshop, Georgetown University, Washington D.C.

CARPENTER D.E., MISRA V. (1991) The expression of exogenous genes by bovine herpesvirus-1 using the BHV-1 VP8 promoter. 10A-16 p.233 Abstr. 16th International Herpesvirus Workshop. Pacific Grove, California.

CARTER J.J., WEINBERG A.D., POLLARD A., REEVES R., MAGNUSON J.A., MAGNUSON N.S. (1989) Inhibition of T-lymphocyte mitogenic responses and effects on cell functions by bovine herpesvirus 1. *J. Virol.* **63**:1525-1530.

CASTRUCCI G., WADA E.M., RANUCCI S., FRIGERI F., CILLI V., PEDINI P., TWSEI B., ARUSH M.A. (1980) Reactivation of latent infection by infectious bovine rhinotracheitis virus in calves. *Microbiologica.* **3**:307-318.

CHANH T.C., KENNEDY R.C., KANDA P. (1988) Synthetic peptides homologous to HIV transmembrane glycoprotein suppress normal human lymphocyte blastogenic response. *Cell. Immunol.* **111**:77-86.

CHASE C.C.L., CARTER-ALLEN K., LETCHWORTH G.J. (1989) The effect of bovine herpesvirus type 1 glycoproteins gI and gIII on herpesvirus infections. *J. Gen. Virol.* **70**:1561-1569.

CHASE C.C.L., CARTER-ALLEN K., LOHFF C., LETCHWORTH G.J. (1990) Bovine cells expressing bovine herpesvirus 1 (BHV-1) glycoprotein IV resist infection by BHV-1, herpes simplex virus, and pseudorabies virus. *J. Virol.* **64**:4866-4872.

CIANCIOLO G.J., COPELAND T.D., OROSZLAN S., SNYDERMAN R. (1985) Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. *Science.* **230**:453-455.

CLARK A. (1987) Herpesvirus vector developed for use in genetic studies. *Research Resources Reporter.* **11**:1-5.

COPELAN E.A., RINEHART J.J., LEWIS M., MATHES L., OLSEN R., SAGONE A. (1983) The mechanism of retrovirus suppression of human T cell proliferation in vitro. *J Immunol.* **131**:2017-2020.

CUMMINS J.M., ROSENQUIST B.D. (1977) Effect of hydrocortisone on the interferon response of calves infected with infectious bovine rhinotracheits virus. *Am. J. Vet. Res.* 38:1163-1166.

DAMIAN R.T. (1989) Molecular mimicry: parasite evasion and host defense. *Curr. Top. Microbiol. Immunol.* 145:101-115.

DESROSIERS R.C., KAMINE J., BAKKER A., SILVA D., WOYCHIK R.P., SAKAI D.D., ROTTMEN F.M. (1985) Synthesis of bovine growth hormone in primates by using a herpesvirus vector. *Mol. Cell. Biol.* 5:2796-2803.

ENGELS M. GIULIANI C., WILD P., BECK T.B., LOEPFE E., WYLER R. (1986) The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization. *Virus Res.* 6:57-73.

ENGELS M., STECK F., WYLER R. (1981) Comparison of the genomes of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis virus strains by restriction endonuclease analysis. *Arch. Virol.* 67:169-174.

ESPOSITO J.J., MURPHY F.A. (1989) Infectious recombinant vectored virus vaccines. *Adv. Vet. Sci. Comp. Med.* 33:195-247.

FEHLER F., HERRMANN J., METTENLEITER T.C., KEIL G.M. (1991) Glycoprotein IV of bovine herpesvirus 1 is essential for viral replication and modulates efficiency of penetration, egress and interference. 6B-07 p.139 Abstr. 16th International Herpesvirus Workshop. Pacific Grove, California.

FILION L.G., MCGUIRE R.L., BABIUK L.A. (1983) Nonspecific suppressive effect of bovine herpesvirus type 1 on bovine leukocyte functions. *Inf. Imm.* 42:106-112.

FITZPATRICK D.R., BABIUK L.A., ZAMB T.J. (1989) Nuclotide sequence of bovine herpesvirus type 1 glycoprotein gIII, a structural model for gIII as a new member of the immunoglobulin suprfamily, and implications for the homologous glycoproteins of other herpesviruses. *Virology.* 173:46-57.

FITZPATRICK D.R., SNIDER B., MCDOUGALL L., BESKORWAYNE T., BABIUK L.A., ZAMB T.J., BIELEFELDT OHMANN H. (1990a) Molecular mimicry: a herpes virus glycoprotein antigenically related to cell-surface glycoprotein expressed by macrophages, polymorphonuclear leukocytes, and platelets. *Immunology.* **70**:504-512.

FITZPATRICK D.R., ZAMB T.J., BABIUK L.A. (1990b) Expression of bovine herpesvirus type 1 glycoprotein gI in transfected bovine cells induces spontaneous cell fusion. *J. Gen. Virol.* **71**:1215-1219.

FORMAN A.J., BABIUK L.A. (1982) Effect of infectious bovine rhinotracheitis virus infection of bovine alveolar macrophage function. *Inf. Imm.* **35**:1041-1047.

FRERICHS G.N., WOODS D.B., LUCAS M.H., SANDS J.J. (1982) Safety and efficacy of live and inactivated infectious bovine rhinotracheitis vaccines. *Vet. Rec.* **111**:116-122.

FUJINAMI, R.S., OLDSTONE M.B.A. (1985) Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for outoimmunity. *Science* . **230**:1043-1045

FUJINAMI, R.S., OLDSTONE M.B.A., WROBLEWSKA Z., FRANKE M.E., KOPROWSKI H. (1983) Molecular mimicry in virus infection: cross reaction in measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. *Proc. Natl. Acad. Sci. USA.* **80**:2346-2350

FULLER A.O., SANTOS R.E., SPEAR P.G. (1989) Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J. Virol.* **63**:3435-3443.

GAMMON G., SHASTRI N., COGSWELL J., WILBUR S., SADEGH-NASSERI S., KRZYCH U., MILLER A., SERCARZ E. (1987) The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol. Rev.* **98**:53-73.

GEORGE L.W., ARDANS A., MIHALYI J. GUERRA M.R. (1988) Enhancement of infectious bovine keratoconjunctivitis by modified-live infectious bovine rhinotracheitis virus vaccine. *Am. J. Vet. Res.* **49**:1800-1806.

GHRAM A., REDDY P.G., BLECHA F., MINOCHA H.C. (1989) Effects of bovine respiratory disease viruses and isoprinosine on bovine leukocyte function in vitro. *Vet. Microb.* **20**:307-314.

GIBBS, E.P.J., RWEYEMAMU M.M. (1977) Bovine herpesviruses. Part 1. *Vet. Bull.* **47**: 317-343.

GRAHAM F.L., VAN DER EB A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* **52**:456-467.

HARRELL R.A., CIANCIOLO G.J., COPELAND T.D., OROSZLAN S., SNYDERMAN R. (1986) Suppression of the respiratory burst of human monocytes by a synthetic peptide homologous to envelope proteins of human and animal retroviruses. *J. Immunol.* **136**:3517-3520.

HAYES M.K., ROCK D.L. (1989) Characterization of a putative latency-related protein of BHV-1. p.49 Abstr. 14th International Herpesvirus Workshop. Nyborg Strand, Denmark.

HEROLD B.C., WUDUNN D., SOLTYS N., SPEAR P.G. (1991) Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.* 65:1090-1098.

HOLLAND T.C., LERCH R.J., EARHART K. (1988) The cytoplasmic domain of herpes simplex virus type 1 glycoprotein C is required for membrane anchoring. *J. Virol.* **62**:1753-1761.

HOMAN E.J., EASTERDAY B.C. (1980) Isolation of bovine herpesvirus-1 from trigeminal ganglia of clinically normal cattle. *Am. J. Vet. Res.* **141**:1212-1213.

HOROHOV D.W., ROUSE B.T. (1986) Virus-induced immunosuppression. In: Veterinary Clinics of North America: Small Animal Practice. D. Ramanauskas, ed. W.B. Saunders, Co., Philadelphia, PA. p.1097-1127..

HOROHOV D.W., WYCOFF J.H., MOORE R.N., ROUSE B.T. (1986) Regulation of herpes simplex virus-specific cell-mediated immunity by a specific suppressor factor. *J. Virol.* **58**, 331.

HUEMER H.P., LAMBRIS J., LARCHER C., ENQUIST L., BABIUK L., EISENBERG R., COHEN G., FRIEDMAN H.M. (1991) Binding of complement component C3 to HSV-1 gC and gC homologs of PRV and BHV-1 shows species specificity. 6A-07 p.132 Abstr. 16th International Herpesvirus Workshop. Pacific Grove, California.

HUTCHINGS D.L., CAMPOS M., QUALTIERE L., BABIUK L.A. (1990a) Inhibition of antigen-induced and interleukin-2-induced proliferation of bovine peripheral blood leukocytes by inactivated bovine herpesvirus 1. *J. Virol.* **64**:4146-4151.

HUTCHINGS D.L., VAN DRUNEN LITTEL-VAN DEN HURK S., BABIUK L.A. (1990b) Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cattle. *J. Virol.* **64**:5114-5122.

ISRAEL B.A., MARSHALL R.L., LETCHWORTH G.J. (1988) Epitope specificity and protective efficacy of the bovine immune response to bovine herpesvirus-1 glycoprotein vaccines. *Vaccine.* **6**:349-356.

IWASAKA T., SHERIDAN J.F., AURELIAN L. (1983) Immmunity to herpes simplex virus type 2: recurrent lesions are associated with the induction of suppressor cells and soluble suppressor factors. *Infect. Immun.* **42**:955-964.

JANEWAY C.A. (1989) Immunotherapy by peptides? *Nature.* **341**:482-483.

JONES C., DELHON G., BRATANICH A., KUTISH G., ROCK D. (1990) Analysis of the transcriptional promoter which regulates the latency-related transcript of bovine herpesvirus 1. *J. Virol.* **64**:1164-1170.

KAHRS R. F. (1977) Infectious bovine rhinotracheitis: A review and update. *J. Am. Vet Med. Assc.* **171**:1055-1064.

KEIL G.M., WEIDEMANN S., BENINGA J. (1989) Size variability of glycoprotein IV of bovine herpesvirus 1. p.136 Abstr. 14th International Herpesvirus Workshop. Nyborg Strand, Denmark.

KIT S., KIT M. (1990) Gene-deleted IBRV marker vaccine. Letter. *Vet. Rec.* **127**:363-364.

KIT S., KIT M., DIMARCHI R.D., LITTLE S.P., GALE C. (1991) Modified-live infectious bovine rhinotracheitis virus vaccine expressing monomer and diver forms of foot-and-mouth disease capsid protein epitopes on surface of hybrid virus particles. *Arch. Virol.* **120**:1-17.

KIT S., KIT M., MCCONNELL S. (1986) Intramuscular and intravaginal vaccination of pregnant cows with thymidine kinase-negative, temperature-resistant infectious bovine rhinotracheitis virus (bovine herpes virus 1). *Vaccine.* 4:55-61.

KIT S., QAVI H., GAINES J.D., BILLINGSLEY P., MCCONNELL S. (1985) Thymidine kinase-negative bovine herpesvirus type 1 mutant is stable and highly attenuated in calves. *Arch. Virol.* **86**:63-83.

KRZYCH U., FOWLER A.V., SERCARZ E.E., (1985) Repertoires of T cells directed against a large protein antigen, Beta-galactosidase: II. Only certain T helper or T suppressor cells are relevant in particular regulatory interactions. *J. Exp. Med.* **162**:311-323.

KRZYCH U., SERCARZ E. (1986) The relationships between Ts-inducing and Th and Tp-inducing determinants on a large protein antigen. *Develop. Biol. Standard.* **63**:41-51.

LABOISSIERE I., SIMARD C., TRUDEL M. (1990) Location on the genome and partial characterization of the gII glycoprotein from bovine herpesvirus type 1. p.234 Abstr. 15th International Herpesvirus Workshop. Georgetown University, Washington D.C.

LAWRENCE W.C., D'URSO R.C., KUNDEL C.A., WHITBECK C., BELLO L.J. (1986) Map location of the gene for a 130,000-dalton glycoprotein of bovine herpesvirus 1. *J. Virol.* **60**:405-414.

LAWRENCE W.C., WHITBECK J.C., BELLO L.J. (1990) Bovine herpesvirus 1 recombinant expresses vesicular stomatitis virus glycoprotein G which is not transported to the cell surface. p.432 Abstr. 15th International Herpesvirus Workshop. Georgetown University, Washington D.C.

LIANG X., BABIUK L.A., VAN DRUNEN LITTEL-VAN DEN HURK S., FITZPATRICK D.R., ZAMB T.J. (1991a) Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. *J. Virol.* **65**:1124-1132.

LIANG X., BABIUK L.A., ZAMB T.J. (1991b) An in vivo study of a glycoprotein gIII negative bovine herpesvirus 1 (BHV-1) mutant expressing B-galactosidase: evaluation of the role of gIII in virus infectivity and its use as a vaccine vector. #239 p. 42 Abstr. 72nd Ann. Mtg. Conf. Res. Workers Anm. Dis., Chicago IL.

LITTLE S.P., JOFFRE J.T., COURTNEY R.J., SCHAFFER R.A. (1981) A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. *Virology.* **115**:149-160.

LO R.Y.C., SHEWEN P.E., STRATHDEE C.A., GREER C.N. (1985) Cloning and expression of the leukotoxin gene of Pasteurella haemolytica A1 in Escherichia coli K-12. *Infect. Immun.* **50**:667-671.

LO R.Y.C., STRATHDEE C.A., SHEWEN P.E. (1987) Nucleotide sequence of the leukotoxin genes of Pasteurella haemolytica A1. *Infect. Immun.* **55**:1987-1996.

LUDWIG G.V., LETCHWORTH G.J. (1987) Temporal control of bovine herpesvirus 1 glycoprotein synthesis. *J. Virol.* **61**:3292-3294.

LUDWIG H. (1983) Bovine herpesviruses. In: The Herpesviruses Vol. 2. B. Roizman, ed. Plenum Press, New York, NY. pp.135-214.

MANIATIS T., FRITSCH E.F., SAMBROOK J. (1982) Isolation of highmolecular-weight, eukaryotic DNA from cells grown in tissue culture. In: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York. pp.280-281.

MARKSON L.B., DARBYSHIRE J.H. (1966) The reaction of calves to experimental infection with the oxford strain of infectious bovine rhinotracheitis virus. *Br. Vet. J.* **122**:522-529.

MARSHALL R.L., ISRAEL B.A., LETCHWORTH G.J., (1988) Monoclonal antibody analysis of bovine herpesvirus-1 glycoprotein antigenic areas relevant to natural infection. *Virology* **165**:338-347.

MARSHALL R.L., RODRIQUEZ L.L., LETCHWORTH G.J. (1986) Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus 1) by biochemical and immunological methods. *J. Virol.* **57**:745-753.

MAYFIELD J.E., GOOD P.J., VANOORT H.J., CAMPBELL A.R., REED D.E. (1983) Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (Cooper Strain). *J. Virol.* **47**:259-264.

MCINTYRE R.W. (1954) Experimental studies of acute upper respiratory infection in calves. *J. Am. Vet. Med. Assc.* **125**:471-472.

MCKERCHER D.G., MOULTON J.E., KENDRICK J.W., SAITO J. (1955) Recent developments on upper respiratory disease of cattle. *Proc. Annu. Meet. US Anim. Health Assoc.* **59**:151-172.

METTENLEITER T.C., ZSAK L., ZUCKERMANN F., SUGG N., KERN H., BEN-PORAT T. (1990) Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. *J. Virol.* **64**:278-286.

METZLER A.E., MATILE H., GASSMANN U., ENGELS M., WYLER R. (1985) European isolates of bovine herpesvirus 1: A comparison of restriction endonuclease sited, polypeptides, and reactivity with monoclonal antibodies. *Arch. Virol.* **85**:57-69.

METZLER A.E., SCHUDEL A.A., ENGELS M. (1986) Bovine herpesvirus 1: molecular and antigenic characteristics of variant viruses isolated form calves with neurological disease. *Arch. Virol.* **87**:205-217.

MILLER-EDGE M., SPLITTER G. (1986) Patterns of bovine T cell-mediated immune responses to bovine herpesvirus 1. *Vet. Imm. Immpath.* **13**:301-319.

MISRA V., BABIUK L.A., DARCEL C.L.Q. (1983) Analysis of bovine herpes virus-type 1 isolated by restriction endonuclease fingerprinting. *Arch. Virol.* **76**:341-354.

MISRA V., BLUMENTHAL R.M., BABIUK L.A. (1981) Proteins specified by bovine herpesvirus 1 (Infectious bovine rhinotracheitis virus). *J. Virol.* **40**:367-378.

MISRA V., GILCHRIST J.E., WEINMASTER G., QUALTIERE L., VAN DEN LITTEL-VAN DEN HURK S., BABIUK L.A. (1982) Herpesvirus-Induced "Early" Glycoprotein: Characterization and possible Role in Immune Cytolysis. *J. Virol.* **43**:1046-1054.

MISRA V., NELSON R., SMITH M. (1988) Sequence of a bovine herpesvirus type-1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. *Virology.* **166**:542-549.

MOORE K.W., VIEIRA P., FIORENTINO D.F., TROUNSTINE M.L., KHAN T.A., MOSMANN T.R. (1990) Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science.* **248**:1230-1234.

NARITA M., INUI S., NAMBA K., SHIMIZU Y. (1976) Trigeminal ganglionitis and encephalitis in calves intranasally inoculated with infectious bovine rhinotracheitis virus. *J. Comp. Pathol.* **86**:93-100.

NELSON R., ADACHI A., CHISHOLM H., MISRA V. (1989) Temperature-sensitive mutants of bovine herpesvirus type 1: Mutants which make unaltered levels of 'early' glycoproteins but fail to synthesize a 'late' glycoprotein. *J. Gen. Virol.* **70**:125-132

NEMEROW G.R., HOUGHTEN R.A., MOORE M.D., COOPER N.R. (1989) Identification of an epitope in the major envelope protein of Epstein-Barr virus that mediates viral binding to the B lymphocyte EBV receptor (CR2). *Cell.* **56**:369-377.

NYAGA P.N., MCKERCHER D.G. (1980) Pathogenesis of bovine herpesvirus-1 (BHV-1) infections: interactions of the virus with peripheral bovine blood cellular components. *Comp. Immunol. Microbiol. Infect. Dis.* **2**:587-602.

OKAZAKI K., MATSUZAKI T., SUGAHARA Y., OKADA J., HASEBE M., IWAMURA Y., OHNISHI M., KANNO T., SHIMIZU M., HONDA E., KONO Y. (1991) BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparinlike moiety on the cell surface. *Virology.* **181**:666-670.

OROSZ C.G., ZINN N.E., OLSEN R.G., MATHES L.E. (1985) Retrovirus- mediated immunnosuppression: I. FeLV-UV and specific FeLV proteins alter T lymphocyte behavior by inducing hyporesponsiveness to lymphokines. *J. Immunol.* **134**:3396-3403.

OWEN L.J., FIELD H.J. (1988) Genomic localization and sequence analysis of the putative bovine herpesvirus-1 DNA polymerase gene. *Arch. Virol.* **98**:27-38.

PASTORET P.P., BABIUK L.A., MISRA V., GRIEBEL P. (1980) Reactivation of temperature-sensitive and non-temperature-sensitive infectious bovine rhinotracheitis vaccine virus with dexamethasone. *Infect. Immun.* **29**:483-488.

ROCK D.L., BEAM S.L., MAYFIELD J.E. (1987) Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. *J. Virol.* **61**:3827-3831.

ROIZMAN B., JENKINS F.J. (1985) Genetic engineering of novel genomes of large DNA viruses. *Science.* **229**:1208-1214.

ROIZMAN B., KOZAK M., HONESS R.W., HAYWARD G. (1974) Regulation of herpesvirus macromolecular synthesis: evidence for multi-level regulation of HSV-1 RNA and protein synthesis. *Cold Spring Harbor Symp. Quant. Biol.* **39**:687-701.

ROIZMAN B., MEIGNIER B., NORRILD B., WAGNER J.L. (1984) Bioengineering of herpes simplex virus variants for potential use as live virus vaccines. In: Modern Approaches to Vaccines. R.M. Chanock and R.A. Lerner, eds. Cold Springs Harbor Laboratory, Cold Springs Harbor, New York. pp 275-281.

ROSSI C.R., KIESEL G.K. (1977) Susceptibility of bovine macrophage and tracheal ring cultures to bovine viruses. *Am. J. Vet. Res.* 38:1705-1708.

SALMAN M.D., KING M.E., ODDE K.G., MORTIMER R.G. (1991a) Annual disease incidence in Colorado cow-calf herds participating in rounds 2 and 3 of the National Animal Health Monitoring System from 1986 to 1988. *J. Am. Vet. Med. Assc.* 6:962-967.

SALMAN M.D., KING M.E., ODDE K.G., MORTIMER R.G. (1991b) Annual costs associated with disease incidence and prevention in Colorado cow-calf herds participating in rounds 2 and 3 of the National Animal Health Monitoring System form 1986 to 1988. *J. Am. Vet. Med. Assoc.* 6:968-973.

SCHOLLER J. (1988) Construction of novel eukaryotic transfection-vector, pJNL-1. *Nuc. Acids Res.* **16**:769.

SCHWARTZ M., WALTENBAUGH C., DORF M., CESLA R., SELA M., BENACERRAF B. (1976) Determinants of antigenic molecules responsible for genetically controlled regulation of immune responses. *Proc. natl. Acad. Sci. U.S.A.* **73**:2862-2866.

SCHWYZER M. (1991) Bovine herpesvirus genome map. p.295 Diagram. 16th International Herpesvirus Workshop. Pacific Grove, California.

SEAL B.S., ST. JEAOR S.C., TAYLOR R.E.L. (1985) Restriction endonuclease analysis of bovine herpesvirus 1 DNA and nucleic acid homology between isolates. *J. gen Virol.* **66**:2787-2792.

SERCARZ E., KRZYCH U. (1991) The distinctive specificity of Ag-specific suppressor T cell. *Immunol. Today.* **12**:111-118.

SERCARZ E.E., YOWELL R.L., TURKIN D., MILLER A. ARANEO B. A., ADORINI L. (1978) Different functional specificity repertoires for suppressor and helper T cells. *Immunol. Rev.* **39**:108136.

SHEWEN P.E., WILKIE B.N., (1982) Cytotoxin of Pasteurella haemolytica acting on bovine leukocytes. *Infect. Immun.* **35**:91-94.

SHROYER E.L., EASTERDAY B.C. (1968) Studies on the pathogenesis of infectious bovine rhinotracheitis following aerosol exposure. *Cornell Vet.* **58**:442-461.

SMITH P.C., NUSBAUM K.E., KWAPIEN R.P., STRINGFELLOW D.A. DRIGGERS K. (1990) Necrotic oophoritis in heifers vaccinated intravenously with infectious bovine rhinotracheitis virus vaccine during estrus. *Am J. Vet. Res.* **51**:969-972.

SOLOMON K.A., ROBBINS A.K., WHEALY M.E., ENQUIST L.W. (1990) The putative cytoplasmic domain of the pseudorabies virus envelope protein gIII, the herpes simplex virus type I glycoprotein C homolog is not required for normal export and localization. *J. Virol.* **64**:3516-3521.

STRATHDEE C.A., LO R.Y.C. (1989) Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the Pasteurella haemolytica leukotoxin determinant. *J. Bacteriol.* **171**:916-928.

TIKOO S.K., FITZPATRICK D.R., BABIUK L.A., ZAMB T.J. (1990) Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. *J. Virol.* **64**:5132-5142.

TOWNSEND A., BODMER H. (1989) Antigen recognition by class I-restricted T lymphocytes. *Ann. Rev. Immunol.* **7**:601-624.

TREPANIER P., SEQUIN C., BASTIEN Y., BOULAY G., LUSSIER G., TRUDEL M. (1985) Hemagglutinating acitvity associated with bovine herpesvirus type 1. *Vet. Micro.* **10**:517-523.

TRUDEL M., SEGUIN C., NADON F., BOULAY G., TREPANIER P., LUSSIER G. (1987). Hemagglutination inhibition and virus neutralizing response of rabbits inoculated with bovine herpesvirus 1 subunit vaccine. *Vet. Micro.* **13**:107-119.

TRUDEL M., BOULAY G., SEGUIN C., NADON F., LUSSIER G. (1988) Control of infectious bovine rhinotracheitis in calves with a BHV-1 subunit-ISCOM vaccine. *Vaccine.* **6**:525-529.

VAN DER MAATEN M.J., MILLER J.M. (1985a) Ovarian lesions in heifers exposed to infectious bovine rhinotracheitis virus by non-genital routes on the day after breeding. *Vet. Microbiol.* **10**:155-163.

VAN DER MAATEN M.J., MILLER J.M., WHETSTONE C.A. (1985b) Ovarian lesions induced in heifers by intravenous inoculation with modified-live infectious bovine rhinotracheitis virus on the day after breeding. *Am. J. Vet. Res.* **46**:1996-1999.

VAN DRUNEN LITTEL-VAN DEN HURK S., BABIUK L. A. (1985c) Effect of tunicamycin and monensin on biosynthesis, transport and maturation of bovine herpesvirus type-1 glycoproteins. *Virology.* **143**:104-118.

VAN DRUNEN LITTEL-VAN DEN HURK S., BABIUK L.A. (1986) Synthesis and processing of bovine herpesvirus 1 glycoproteins. *J. Virol.* **59**:401-410.

VAN DRUNEN LITTEL-VAN DEN HURK S., GIFFORD G.A., BABIUK L.A. (1990a) Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. *Vaccine.* **8**:358-368.

VAN DRUNEN LITTEL-VAN DEN HURK S., HUGHES G., BABIUK L.A. (1990b) The role of carbohydrate in the antigenic and immunogenic structure of bovine herpesvirus type 1 glycoproteins gI and gIV. *J. Gen. Virol.* **71**:2053-2063.

VAN DRUNEN LITTEL-VAN DEN HURK S., PARKER M.D., FITZPATRICK D.R., ZAMB T.J., VAN DEN HURK J.V., CAMPOS M., HARLAND R., BABIUK L.A. (1991) Expression of bovine herpesvirus 1 glycoprotein gIV by recombinant baculovirus and analysis of its immunogenic properties. *J. Virol.* **65**:263-271.

VISCIDI R.P., KUMUDINI M. ,LEDERMAN H.M. ,FRANKEL A.D. (1989) Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. *Science.* **246**:1606-1608.

WEINMASTER G.A. MISRA V., MCGUIRE R., BABIUK L.A., DECLERCQ E. (1982) Bovid herpesvirus type-1 (infectious bovine rhinotracheitis virus)-induced thymidine kinase. *Virology.* **118**:191-201.

WESTERMAN K., SKARE I.B. (1990) Characterization of an origin of replication sequence in bovine herpesvirus 1 DNA. p.126 Abstr. 15th International Herpesvirus Workshop. Georgetown University, Washington D.C.

WHETSTONE C.A., MILLER J.M. (1989a) Dual latency in BHV-1 infections. P37, p.69. Abstr. 70th Ann. Mtg. Conf. Res. Workers Anm. Dis., Chicago IL.

WHETSTONE C.A., MILLER J.M., SEAL B.S., BELLO L.J., LAWRENCE W.C. (1990a) Experimental inoculation of cattle with a TK- deletion mutant of bovine herpesvirus 1 (BHV 1) p. 324. Abstr. 15th International Herpesvirus Workshop. Georgetown University, Washington D.C.

WHETSTONE C.A., MILLER J.M., SEAL B.S., BELLO L.J., LAWRENCE W.C. (1990b) TK- bovine herpesvirus 1 does not cause abortion, establishes latency and is reactivated after DX. #249 p.45 Abstr. 71st Ann. Mtg. Conf. Res. Workers Anm. Dis. Chicago, IL

WHETSTONE C.A., MILLER J.M., VAN DER MAATEN M.J. (1989b) Establishment of latent infection with second strain of bovine herpesvirus 1 in animals already latently infected. p.64 Abstr. 14th International Herpesvirus Workshop. Nyborg Strand, Denmark.

WHETSTONE C.A., WHEELER J.G., REED D.E. (1986) Investigation of possible vaccine-induced epizootics of infectious bovine rhinotracheitis, using restriction endonuclease analysis of viral DNA. *Am. J. Vet. Res.* **47**:1789-1795.

WHETSTONE C.A., WHEELER J.G., REED D.E.. (1986) Investigation of possible vaccine-induced epizootics of infectious bovine rhinotracheitis, using restriction endonuclease analysis of viral DNA. *Am. J. Vet. Res.* **46**:1789-1795.

WHITBECK J.C., BELLO L.J., LAWRENCE W.C. (1988) Comparison of the bovine herpesvirus 1 gI gene and the herpes simplex virus type 1 gB gene. *J. Virol.* **62**:3319-3327.

WIRTH U.V., GUNKEL K., ENGELS M., SCHWYZER M. (1989) Spatial and temporal distribution of Bovine herpesvirus 1 transcripts. *J. Virol.* 63:4882-4889.

WIRTH U.V., SCHWYZER M., METZLER A.E., ENGELS M., WYLER R. (1989) Immediate-early genes of bovine herpesvirus 1 (BHV-1). p.220 14th International Herpesvirus Workshop. Nyborg Strand, Denmark.

WONG G.G., WITEK J.S., TEMPLE P.A., WILKENS K.M., LEARY A.C., LUXENBERG D.P., JONES S.S., BROWN E. L. , KAY R.M., ORR E.C., SHOEMAKER C., GOLDE D.W., KAUFMAN R.J., HEWICK R.M., WANG E.A., CLARK S.C. (1985) Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science.* 228:810-815.

WUDUNN D., SPEAR P.G. (1989) Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* 63:52-58.

WYLER R., ENGELS M. , SCHWYZER M. (1989) Infectious bovine rhinotracheitis/vulvovaginitis (BHV-1). In: Herpesvirus diseases of cattle, horses, and pigs. G. Wittmann ed. Kluwer Academic Publishers, Norwell MA. pp.1-72.

YATES W.D.G. (1982) A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Can. J. Comp. Med.* 46:225-263.

APPENDIX A

Restriction endonuclease analysis and cloning of the bovine herpesvirus-1 genome subtype 2.

The diagnosis of a bovine herpesvirus-1 infection has relied upon the antigenic characteristics of the virus in fluorescent-antibody tests, ELISA, or radioimmunoassays. Restriction endonuclease analysis has enabled the further definition of BHV-1 into subtypes. Characteristic differences exist between the respiratory strain or subtype 1, the genital strain or subtype 2, and neurologic strain or subtype 3 forms of BHV-1 (Engels, Steck, & Wyler, 1981; Engels et al., 1986; Metzler, Schudel, & Engels, 1986; Misra, Babiuk, & Darcel, 1983). We have compared the restriction endonuclease pattern of a field isolate of BHV-1 (isolated by Dr. J. Storz) to the ATCC Cooper strain. The field strain shows distinct fragment polymorphisms which are characteristic of the infectious pustular vulvovaginitis isolates.

Georgia bovine kidney (GBK) cells were used for propagating and titering virus stock and were cultivated in Dulbecco's Modified Eagle's medium (DMEM) with sodium bicarbonate (Sigma Chemical Company, St. Louis, MO) supplemented with 20mM HEPES, 5% fetal calf serum, 100 U/ml penicillin and 100 µg/ml of streptomycin. The Cooper strain of BHV-1 (ATCC and Field isolate) was propagated in cells at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C until the cytopathic effect reached 100%. The supernate was clarified of cell debris by low speed centrifugation and then layered over a 20% sucrose cushion and centrifuged for 2 hours at 100,000 x g. The

virions were resuspended in $T_{10}E_1$ (10mM TrisHCl, 10mM EDTA, pH=8.0), and 100 ug/ml proteinase K and 0.5% Sarkosyl were added and the mixture allowed to incubated at 42° C overnight. The DNA was then extracted with phenol and ethanol precipitated.

For restriction endonuclease analysis, viral DNA was digested with Hind III, Eco RI, Bam HI, Bgl II, Hpa I, Kpn I, and Pst I according to standard protocols and manufacturers directions (New England Biolabs, Inc., Beverly MA). The digested DNA was separated on an 0.8% agarose gel in Tris Phosphate buffer (0.04 M Tris-phosphate, 0.001M EDTA, pH=8.0) and stained with ethidium bromide.

A library of genomic fragments was constructed using the pUC19Bgl (constructed by K.G. Kousoulas). Viral DNA Hind III fragments were cloned into the corresponding site in the polylinker using standard DNA methodology (Maniatis, Fritsch, & Sambrook, 1982).

Figure A.1 shows the results of the restriction endonuclease analysis of the field strain versus the ATCC Cooper strain when digested with Hind III, Eco RI, Bam HI, Bgl II, Hpa I, and Kpn I. Bands below 1.4 kilobases could not be identified on the stained gels. Fragments which comigrated could not be visually separated except by the intensity of the ethidium bromide staining.

Figure A.2 shows the BHV-1 field strain library in pUC19Bgl. Fragments C and D, sections of the unique short region including the 5' inverted repeat, were not isolated from the ligation pool, but are represented by fragments H, F, and K.

We have compared the restriction endonuclease patterns of a field isolate of BHV-1 to those of the ATCC Cooper strain of BHV-1.

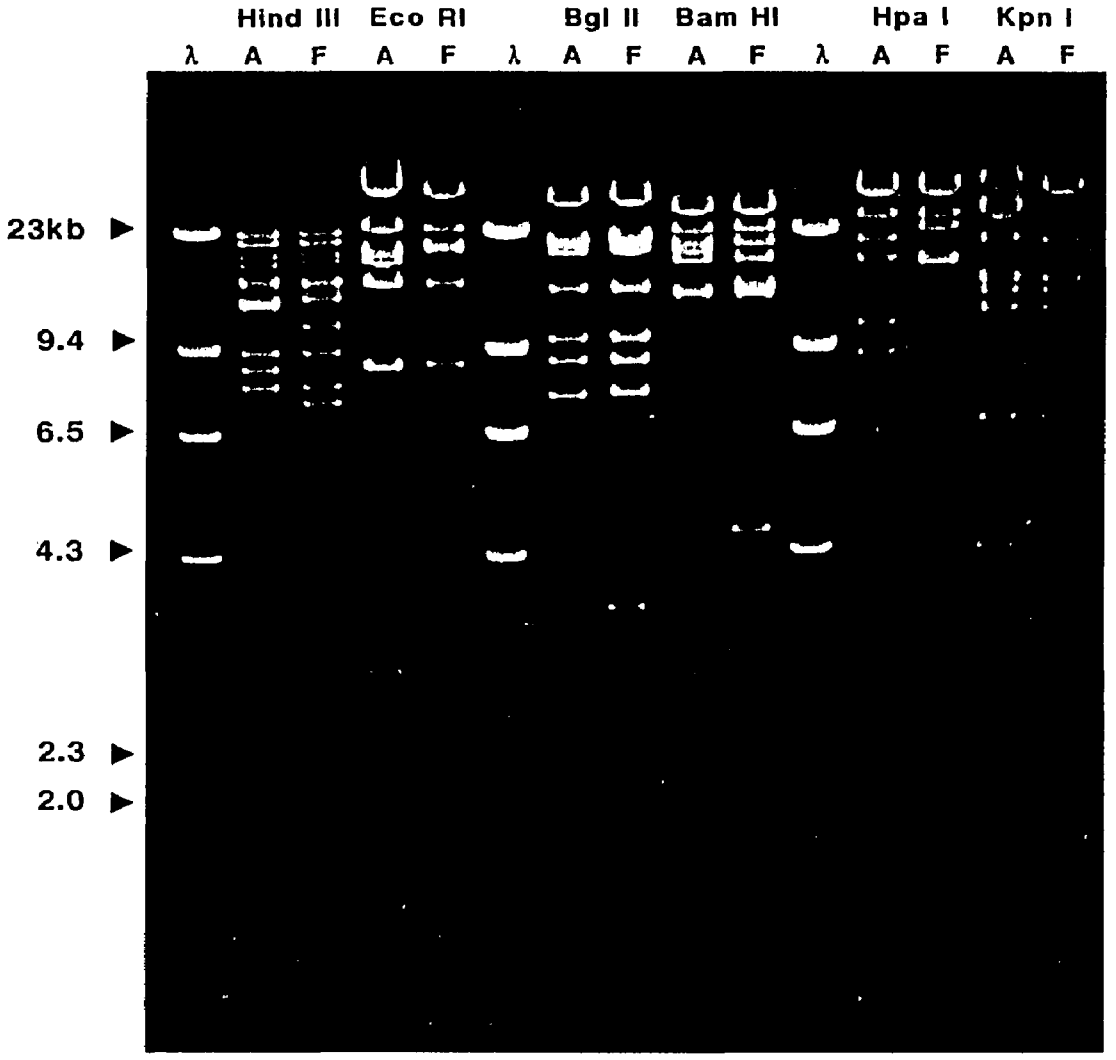

Figure A.1

Restriction endonuclease analysis of the field strain versus the ATCC Cooper strain of BHV-1. Viral DNA was digested with Hind III, Eco RI, Bgl II, Bam HI, Hpa I, and Kpn I. ATCC viral DNA, designated with an"A", was run parallel with field strain viral DNA, designated with an "F".

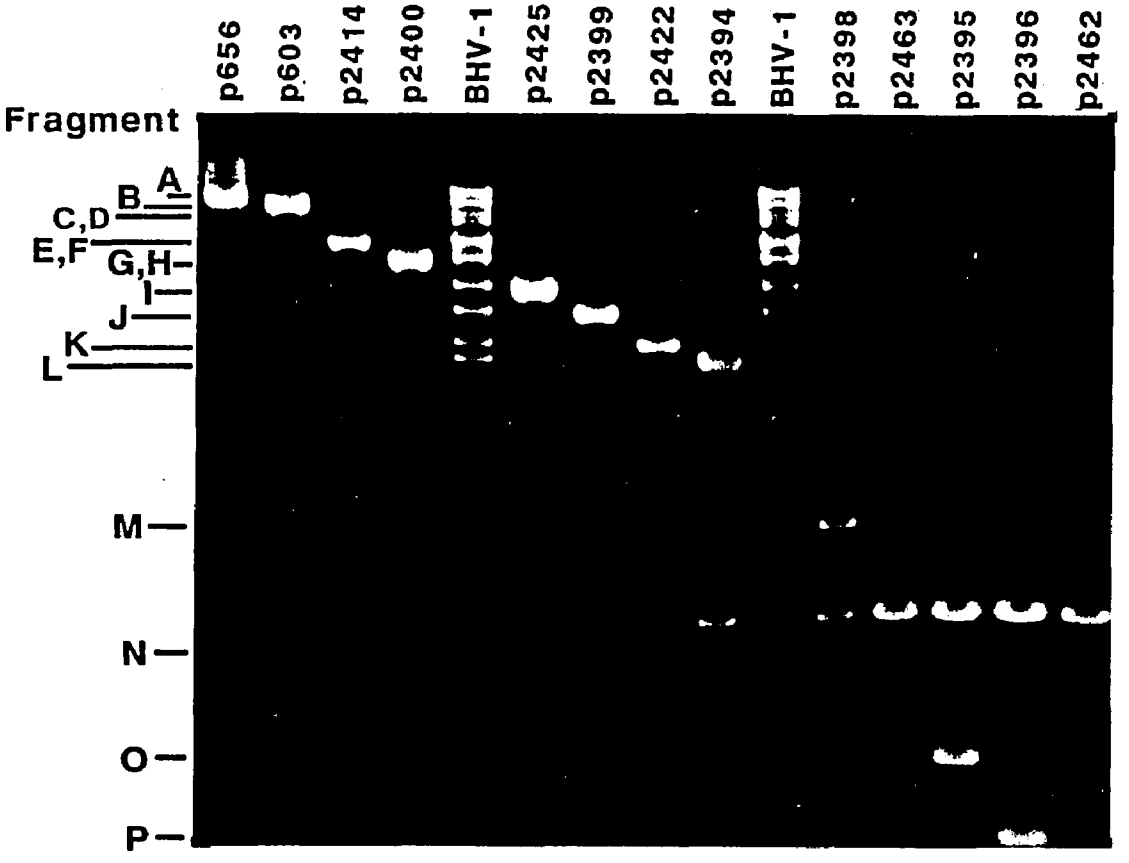

Figure A.2

Bovine herpesvirus-1 field strain viral DNA clonal library in pUC19Bgl. Lanes 5 and 10 are field strain viral DNA digested with Hind III which represent fragments A through P. Plasmids were digested with Hind III to correlate their insert with viral DNA.

The pattern of the field isolate is homologous for the pattern previously published for infectious pustular vulvovaginitis isolates (Seal, St. Jeor, & Taylor, 1985; Metzler et al., 1985; Engels, Steck, & Wyler, 1981). This pattern is distinct for the locations of the ATCC Hind III "I" fragment above its usual location, and the lack of the ATCC Eco RI "G" fragments. Fragments for the Bgl II, Bam HI, Hpa I, and Kpn I digests also differ between the two genomes. Based on distinctly differing patterns of the field strain to the ATCC Cooper strain, and its homologous patterns to the infectious pustular vulvovaginitis form, we are categorizing the field strain as a BHV-1 subtype 2 pattern for restriction endonuclease analysis.

APPENDIX B

Isolation of the lktA gene of Pasteurella haemolytica from pLKT52 using the polymerase chain reaction.

The bovine herpesvirus genome has been used as a vector to express heterologous antigens (Kit et al., 1991). This approach can provide protection against multiple pathogens in the form of a modified live BHV-1 vaccine. The lktA gene of Pasteurella haemolytica has been isolated from a plasmid containing the leukotoxin operon. This represents the initial step to producing a recombinant BHV-1 which would also protect against Pasteurella.

Pasteurella haemolytica serotype 1 is the principal pathogenic bacteria involved in the bovine respiratory disease complex. As part of its virulence, it encodes for a leukotoxin specific for bovine lymphocytes (Shewen & Wilkie, 1982). The leukotoxin operon has been isolated, sequenced, and characterized as the plasmid pLKT52 (Lo et al., 1985; Lo, Strathdee, & Shewen, 1987; Strathdee & Lo, 1989). The lktA gene encodes for the structural component of the leukotoxin. The lktC gene product acts on the LKTA protein to yield an active leukotoxin, which is subsequently secreted by the actions of the lktB and lktD gene products. Because of the closeness of the genes within the operon, isolation of a single gene is difficult using standard cloning methods. We have isolated the lktA gene using the polymerase chain reaction (PCR) and cloned it into a modified pUC19 vector and the transient eukaryotic expression vector, p91023.

Primers for the PCR were produced based on the 5'and 3' published sequences of the lktA gene and are shown in Figure B.1 (Lo, Strathdee, & Shewen, 1987). To facilitate cloning into the p91023 vector, the sequences for the Bgl II and Eco RI restriction sites were incorporated into the 5' and 3' primers, respectively. The pLKT52 plasmid containing the leukotoxin operon (supplied by Dr. R.Y.C. Lo) was cut with Nde I in order to linearize the plasmid and improve the Taq polymerase reaction. The PCR reaction mixture consisted of 200 pmoles of each primer, 1 ng of target template, 50 mM of each dNTP,1X PCR buffer (20mM Tris-HCl pH=8.3, 1.5mM MgCl2, 25mM KCl, 0.05% Tween-20, 100 μg/ml autoclaved gelatin), and 2.5 U of Taq polymerase with enough distilled water to bring the mixture to 50 μl. The Eppendorf Microcycler was programmed to run for 1 second at 96° C, 20 seconds at 53° C, and 3 minutes at 72° C, for 25 cycles. The fragments were then extended for 7 minutes at 72° C.

The resulting fragment corresponded to the predicted 3.4 kilobase length of the lktA gene. The PCR mixture was cleaned with chloroform and precipitated, resuspended in $T_5E_{0.1}$ (5mM Tris-Cl, 0.1 mM EDTA, pH=8.0) and digested with Bgl II and Eco RI, using standard techniques (Maniatis, Fritsch, & Sambrook, 1982). The fragment was then ligated into a modified pUC19 vector (containing a Bgl II restriction enzyme site) and a p91023 vector at their Bgl II-Eco RI sites, and transformed into DH5α cells. The resulting plasmids, p2320 and p2324 in pUC19 and p91023 respectively, were prepared and purified over two cescium chloride gradients. Figure B.2 shows diagnostic restriction enzyme digests for p2320. The 5' end of p2320 was also sequenced to ascertain its exact insertion. The Sequenase Kit

5' PRIMER:

5' ATC AGA TCT TGT GAA ACA ATA TAG AGT TGC CA 3'

3' PRIMER:

5' ATC GAA TTC AGG AGA CAT CCC TTA TGG GA 3'

Figure B.1.

PCR primer sequences for the isolation of the lktA gene from pLKT52.

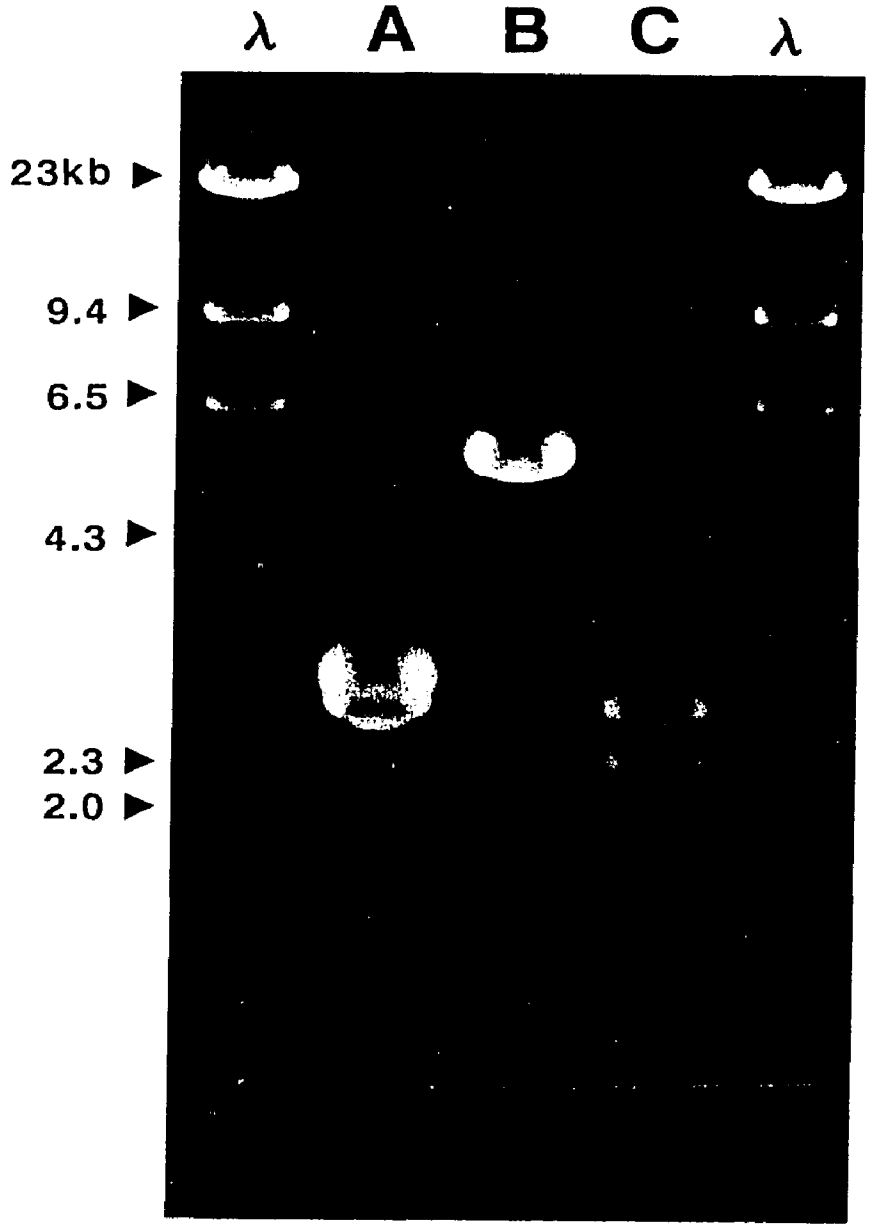

Figure B.2

Restriction enzyme digests of p2320. Lane 1 and 5 are the lambda molecular weight marker. Lane 2 shows the Bgl II and Eco RI digest. Lane 3 shows the diagnostic Cla I digest. Lane 4 shows the Bgl II, EcoRI, and Cla I digests showing Cla I restriciton site within the p2320's insert and not the vector.

(United States Biochemical Corp, Cleveland, OH) was used as directed for denaturing double stranded DNA. The resulting sequence (180 bases) corresponded to that published for the lktA gene.

In order to test for expression of the lktA gene product, p2324 was transfected into COS-7 cells. COS-7 cells at 50% confluency on glass coverslips in 24-well tissue culture plates were transfected with 5 micrograms of plasmid DNA using a modified calcium phosphate precipitation technique (Graham & Van Der Eb, 1973). Plasmid DNA was mixed with 25 µl of 2.5M $CaCl_2$ and the volume brought up to 250 µl with distilled water. An equal volume of 2X HEPES-buffered saline (2XHBS: 280mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES acid, pH=7.05) was added to the DNA mixture with a constant bubbling of air. The solution was incubated at room temperature for 30 minutes. Wells of COS-7 cells were washed once with tris-buffered saline (TBS: 137mM NaCl, 5mM KCl, 1.4mM Na_2HPO_4, 25mM Tris Base, 1.4mM $CaCl_2$, 0.5mM $MgCl_2$, pH=7.5) and incubated for 2 minutes with a 0.5 mg/ml DEAE-dextran (Sigma Chemical Co., St. Louis, MO) solution in TBS, after which the DNA mixture was added. Following a 4 hour incubation at 37°C, the cells were shocked with a 15% glycerol in 1X HBS for 2 minutes and washed with 1x HBS. Fresh media was added and the cells incubated for 40 hours.

Expression of lktA by COS-7 cells was tested by indirect immunofluorescence (IFA). The coverslips were fixed with methanol and blocked with 10% goat serum in PBS for 1 hour at room temperature. Monoclonal antibodies specific for the leukotoxin (supplied by Dr. Corstvet) were diluted 1:2 and 1:10 in PBS with 1% goat serum and incubated with the monolayer for 1 hour at 37° C.

The coverslips were washed in PBS and incubated with FITC conjugated rabbit anti-mouse IgM and IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:50 and incubated for 1 hour at 37° C.

Indirect immunofluorescence could not detect the expression of the LKTA protein in COS-7 cells. This could be due to the lack of expression by p2324, the inaccesability of the expressed protein to the antibody, or the inability of the antibody selected for its activity against the active leukotoxin to detect the structural LKTA component. Despite the inability to detect the expression of LKTA using the p91023 vector and COS-7 cells, the isolation of the structural gene for the leukotoxin provides for the development of a new recombinant viral vaccine which would express an inert form of the protein but could maintain its protective antigenicity.

VITA

Katherine Marie Byrne, the daughter of Maudena Z. Bower and Norman E. Byrne, was born on July 5, 1962 in Frankfort, Indiana. She graduated as Valedictorian from Pattonville High School in Bridgeton, Missouri in May, 1980. In August of 1982, after two years of undergraduate study, she entered the professional curriculum of the University of Missouri, College of Veterinary Medicine. She graduated with a Doctor of Veterinary Medicine degree in May, 1986. She entered private veterinary clinical practice in Salt Lake City, Utah following graduation. She began her Doctor of Philosophy studies in the Department of Veterinary Microbiology and Parasitology at Louisiana State University in January, 1988. She was awarded a Board of Regents Fellowship in September, 1988.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Katherine Marie Byrne

Major Field: Veterinary Medical Sciences

Title of Dissertation: The Role of Bovine Herpesvirus-1 Glycoprotein III in Molecular Pathogenesis and Immunomodulation

Approved:

Konstantin G. Kousoulas
Major Professor and Chairman

Kathleen dela Peña McCorkle
Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

December 16, 1991